CHARLES UNIVERSITY IN PRAGUE

FACULTY OF SCIENCE

Department of Plant Experimental Botany

Isoflavone Synthase: Presence and Activity in Leguminous and Non-leguminous Plants

MASTERS THESIS

Martina Pičmanová

Supervisor: RNDr. David Honys, PhD.

Prague 2010

The present work was carried out at the Laboratory of Pollen Biology at the Institute of Experimental Botany AS CR, Prague.

Declaration

I hereby declare that I completed this masters thesis independently, except where explicitly indicated otherwise, and that it documents my own work, carried out under the guidance of my supervisor RNDr. David Honys, PhD. Throughout, I have properly acknowledged and cited all sources used.

Prague, 24 August 2010 ……..……..

All we need is plant.

Acknowledgements

It is a pleasure to thank my supervisor RNDr. David Honys, PhD. for his patient guidance, valuable comments and useful ideas. I would like to acknowledge my debt to Prof. RNDr.Oldřich Lapčík, Dr., who first introduced me to the fascinating world of isoflavonoids and enabled me to participate in his project. I feel very grateful to all my laboratory colleagues, who were always ready to help, answer any queries and encourage me in every crisis situation. Many thanks are due to Tsuyoshi Nakagawa, who so kindly provided us with the pGWB binary vectors; Ing. Jana Feciková for her guidance on proteomic analysis; Mgr. Edita Drdová, Mgr. David Reňák and RNDr. Jan Petrášek, Ph.D., for their help with transient expression procedures and confocal microscopy; Ing. Petra Mikšátková for taking the HPLC-MS measurements; and Ing. Roman Pleskot for his molecular-modelling art. I would like to express my whole-hearted gratitude to my partner in life, for our intellectually-stimulating and thoughtful discussions, and for his infinite patience. Last but not least, I would like to thank my parents, whose all-embracing support has been essential – and not just for my studies.

This work was supported by the following grants: GAČR 525/06/0864 – Revize taxonomické distribuce isoflavonoidů, Czech Science Foundation; GAČR 525/09/0994 - Chemotaxonomie isoflavonoidů, Czech Science Foundation; LC06004 – Integrovaný výzkum rostlinného genomu, Ministry of Education, Youth and Sports of the Czech Republic.

ABSTRACT

Isoflavone synthase (IFS; CYP93C) plays a key role in the biosynthesis of the plant secondary metabolites, isoflavonoids. These phenolic compounds, which are well-known for their multiple biological effects, are produced mostly in leguminous plants (family Fabaceae). However, at least 225 of them have also been described in 59 other families, without any knowledge of orthologues to hitherto known *IFS* genes from legumes (with the single exception of sugar beet – *Beta vulgaris,* from the family Chenopodiaceae).

In view of these facts, this masters thesis has focused on two main objectives: (1) to identify isoflavone synthase genes in selected leguminous and non-leguminous plants exploiting the PCR strategy with degenerate and non-degenerate primers, and (2) to find a system for the verification of the correct function of these genes.

Our methodology for the identification of *IFS* orthologues was successfully demonstrated in the case of two examined legumes – *Phaseolus vulgaris* L. and *Pachyrhizus tuberosus* (Lam.) Spreng, in the genomic DNA of which the complete *IFS* sequences have been newly identified. To design a procedure for ascertaining the correct function of these genes and others once they have been completely described, a pilot study with IFS from *Pisum sativum* L. (CYP93C18; GenBank number AF532999) was conducted. *CYP93C18* was identified, cloned and introduced into the putative isoflavone pathway-free plant *Arabidopsis thaliana* using Gateway™ Technology. Its correct function was verified at four different levels by: PCR with *IFS*-specific primers (DNA), RT-PCR (RNA), Western blotting (proteins) and HPLC-MS (metabolites). In addition, CYP93C18::GFP fused proteins were transiently expressed in the leaves of *Nicotiana benthamiana,* and the localization of the GFP signal was observed on the endoplasmic reticulum using confocal microscopy, which is consistent with the predicted presence of a signal peptide in the IFS´s N-terminus, as well as with the model of IFS generated *in silico* on the basis of cytochromes P450 homology.

Keywords: cytochrome P450, isoflavone synthase, CYP93C18, *Pisum sativum* L., *Pachyrhizus tuberozus* (Lam.) Spreng, *Phaseolus vulgaris* L., gene over-expression, GFP, endoplasmic reticulum membrane

ABSTRAKT

Isoflavonsynthasa (IFS; CYP93C) hraje klíčovou roli v biosyntéze rostlinných sekundárních metabolitů – isoflavonoidů. Tyto fenolické látky, známé díky širokému spektru svých biologických účinků, jsou produkovány především rostlinami z čeledi bobovitých (Fabaceae). Ačkoliv bylo nejméně 225 isoflavonoidů popsáno i v 59 dalších čeledích, ortolog známých *IFS* z bobovitých rostlin byl doposud popsán pouze v jediné nebobovité rostlině – *Beta vulgaris* (čeleď Chenopodiaceae).

Tato diplomová práce si na základě zmíněných poznatků kladla za cíl (1) identifikovat ortologní geny pro isoflavonsynthasu ve vybraných bobovitých a nebobovitých rostlinách a (2) vytvořit sytém pro ověřování správné funkce těchto genů.

Naše metodika pro identifikaci ortologů *IFS* se osvědčila v případě dvou zkoumaných bobovitých rostlin – *Phaseolus vulgaris* L. a *Pachyrhizus tuberosus* (Lam.) Spreng, v jejichž genomické DNA byly nově identifikovány kompletní sekvence genu pro IFS. Aby bylo možno v budoucnu ověřit správnou funkci těchto a dalších případných genů pro IFS, byla provedena pilotní studie s IFS isolované z *Pisum sativum* L. (CYP93C18; GenBank number AF532999). Gen pro CYP93C18 byl identifikován, klonován s využitím Gateway™ technologie a vnesen do *Arabidopsis thaliana* – rostliny postrádající biosyntetickou dráhu isoflavonoidů. Správná funkce *CYP93C18* v transgenních rostlinách pak byla ověřována na čtyřech úrovních: PCR s primery specifickými k *IFS* (DNA), RT-PCR (RNA), Western bloty (proteiny) a HPLC-MS (metabolity). Zároveň byla potvrzena správná buněčná lokalizace CYP93C18 metodou transientní exprese fúzních proteinů IFS::GFP v listech *Nicotiana benthamiana.* Fluorescenční signál byl konfokálním mikroskopem pozorován na endoplasmatickém retikulu, což odpovídá predikované přítomnosti signálního peptidu na N-konci IFS, stejně jako modelu generovanému *in silico* na základě homologie cytochromů P450.

Klíčová slova: cytochrom P450, isoflavonsynthasa, CYP93C18, *Pisum sativum* L., *Pachyrhizus tuberozus* (Lam.) Spreng, *Phaseolus vulgaris* L., genová overexprese, GFP, membrána endoplasmatického retikula

CONTENTS

Abbreviations 9

1. INTRODUCTION 10

1.1. Aims of the present study 10

2. LITERATURE SURVEY 11

2.1. Isoflavonoid biosynthesis 11

2.1.1. Phenylpropanoid metabolic pathway 11

2.1.2. The biosynthetic pathway leading to the production of isoflavonoids 12

2.2. Isoflavone synthase (IFS) 15

2.2.1. Cytochromes P450 (CYPs) 15

2.2.2. CYP93C 15

2.2.3. Reaction mechanism of IFS 18

2.2.4. Molecular modelling of the IFS active site 19

2.3. 2-hydroxyisoflavanone dehydratase (2-HID) 20

2.4. Isoflavonoids 21

2.4.1. Structure and classification of isoflavonoids 21

2.4.2. Isolation and identification of isoflavonoids 23

2.4.3. Taxonomical distribution of isoflavonoids 23

2.4.4. Biological functions of isoflavonoids in plants 24

2.4.5. Pharmacological effects of isoflavonoids 26

2.5. Metabolic engineering of isoflavonoid biosynthesis 28

3. MATERIAL AND METHODS 30

3.1. Material 30

3.1.1. Plant material 30

3.1.2. Bacterial strains and plasmids 31

3.1.3. Antibiotics 31

3.1.4. Growth media 31

3.1.5. Chemicals 32

3.1.6. Commercial kits for molecular biology 33
3.2. General laboratory equipment used.....33
3.3. Computational programmes and databases.....33
3.4. Methods and procedures 34
3.4.1. Outline of our approach 34
3.4.2. Genomic DNA extraction 35
3.4.3. Identification of isoflavone synthase gene (*IFS*).....35
3.4.3.1. Primers design.....35
3.4.3.2. Polymerase chain reaction (PCR).....36
3.4.3.3. Horizontal agarose gel electrophoresis.....37
3.4.3.4. DNA sequencing.....38
3.4.4. Cloning of fragments obtained with degenerate primers 38
3.4.5. Cloning of putative *IFS* genes from *Phaseolus vulgaris* and *Pachyrhizus tuberosus*.....40
3.4.6. Cloning and over-expression of *CYP93C18* using Gateway™ Technology.....41
3.4.6.1. Directional TOPO® cloning of *CYP93C18*.....41
3.4.6.2. LR recombination reaction 43
3.4.6.3. Transformation of *Agrobacterium tumaefaciens*.....44
3.4.7. Stable expression of CYP93C18.....44
3.4.7.1. Transformation of *Arabidopsis thaliana*.....44
3.4.7.2. Selection of transformants 45
3.4.7.3. Analysis of transgenic *Arabidopsis thaliana*.....45
3.4.7.3.1. Level 1: DNA.....45
3.4.7.3.2. Level 2: RNA.....46
3.4.7.3.3. Level 3: Proteins.....46
3.4.7.3.4. Level 4: Metabolites.....48
3.4.8. Transient expression of CYP93C18.....48
3.4.8.1. Transformation of *Nicotiana benthamiana*.....48
3.4.8.2. Analysis of transgenic *Nicotiana bethamiana*.....49
3.4.9. Modelling of CYP93C18 and computational methods 49

4. RESULTS 50
4.1. Identification of isoflavone synthase genes (*IFS*) 50

4.1.1. Identification of *IFS* in the selected non-leguminous species.....50
4.1.2. Identification of complete *IFS* sequences from *Pachyrhizus* and *Phaseolus* 54
4.1.2.1. Characterization of IFS from *Phaseolus vulgaris* L. 55
4.1.2.2. Characterization of IFS from *Pachyrhizus tuberozus* (Lam.) Spreng. 56
4.2. Pilot study with IFS (CYP93C18) from *Pisum sativum* L.....57
4.2.1. Identification and isolation of *CYP93C18* 57
4.2.2. TOPO® cloning of *CYP93C18* 61
4.2.3. Construction of *CYP93C18* expression clone for stable expression.....63
4.2.4. Construction of *CYP93C18* expression clone for transient expression.....65
4.2.5. Transformation of *Agrobacterium tumaefaciens* 66
4.2.6. *Arabidopsis thaliana* transformation with pGWB2::IFS 67
4.2.7. Verification of correct function of CYP93C18 transgene.....69
4.2.7.1. DNA level 69
4.2.7.2. RNA level 70
4.2.7.3. Protein level 71
4.2.7.4. Metabolite level 73
4.2.8. Visualization of CYP93C18 *in vivo* 77
4.2.9. IFS association with endoplasmic reticulum membrane.....77

5. DISCUSSION 80
5.1. *IFS* was detected in leguminous species only.....80
5.2. CYP93C18 is a functional isoflavone synthase 83
5.3. IFS is localized on the membrane of the endoplasmic reticulum.....86

6. CONCLUSIONS 88

7. REFERENCES.....89

ABBREVIATIONS

CDS	Coding DNA sequence
CHI	Chalcone isomerase
CHS	Chalcone synthase
CYP93C	Subclass 93 of cytochrome P450
D7OMT	Daidzein 7-*O*-methyltransferase
ddH_2O	Double-distilled water
DFR	Dihydroflavonol 4-reductase
ER	Endoplasmic reticulum
F3H	Flavanone 3β-hydroxylase
FNS	Flavone synthase
GFP	Green fluorescent protein
HI4´IOMT	Hydroxyisoflavanone 4´-*O*-methyltransferase;
HPLC-ESI-MS	High performance liquid chromatography-electrospray ionisation- mass spectrometry
IFD	Isoflavone dehydratase
IFS	Isoflavone synthase (gene)
IFS	Isoflavone synthase (protein)
IOMT	Isoflavanone *O*-methyltransferase
LB medium	Luria-Bertani medium
MS medium	Murashige-Skoog medium
NADPH	Nicotinamide adenine dinucleotide phosphate
PCR	Polymerase chain reaction
RNAi	RNA interference
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

1. INTRODUCTION

Amidst the vast number of cytochromes P450, isoflavone synthase (IFS; belonging to the CYP93C subfamily) occupies a special place due to its key role in the biosynthesis of the plant secondary metabolites, isoflavonoids. Although these phenolic compounds are produced predominantly in the Fabaceae family, they have been detected in a further 59 plant families, as far as known to date (Macková *et al*., 2006).

The literature deals extensively with the positive effects of these well-known phytoestrogens on human health, including cancer prevention and the mitigation of menopause symptoms, as well as with the potential risks associated with their consumption (Ososki and Kennelly, 2003; Cornwell *et al.,* 2004). In addition, isoflavonoids have considerable importance for plants themselves, particularly as phytoalexins and chemoattractants in rhizobial symbiosis (Dakora and Phillips, 1996).

Isoflavone synthase is known for its outstanding ability to catalyze both the hydroxylation of the two flavanone precursors – liquiritigenin and naringenin – as well as the critical migration of the aryl group from the C2 to the C3 position on the chromene skeleton of the flavanones. To date, 31 individual IFS-amino-acid sequences, displaying a homology of more than 95%, have been described. Apart from this, the presence of *IFS* (in two isoforms) has been reported only once in the case of only a single non-leguminous species, *Beta vulgaris* (sugar beet) from family Chenopodiaceae (Jung *et al.*, 2000).

1.1. Aims of the present study

In the light of the fact that there is a number of isoflavonoid-producing plant families other than the Fabaceae family and yet we lack any knowledge of the genetic background of their isoflavonoid biosynthesis (with the sole exception of sugar beet), the main objectives of this thesis were formulated as follows:

(1) The identification of isoflavone synthase genes in selected non-leguminous and leguminous plants;

(2) The performance of a pilot study with one of the two known *IFS* genes from *Pisum sativum* L. – namely *CYP93C18*, GenBank accession no. AF532999 (Cooper *et al*., 2005) – in order to develop a system for the verification of the correct function of newly-identified genes orthologous to known *IFS*s. This study also entails the visualization of IFS *in vivo*, thus providing confirmation of its subcellular localization.

2. LITERATURE SURVEY

2.1. Isoflavonoid biosynthesis

Isoflavonoids represent a group of phenolic plant secondary metabolites and are well-known for their multiple beneficial effects to both plant and human health. Their biosynthetic pathway and the unusual manner of their formation are therefore frequently discussed in the literature, in most cases in connection with metabolic engineering.

2.1.1. Phenylpropanoid metabolic pathway

The plant phenylpropanoid pathway is responsible for the production of a broad spectrum of phenolic secondary metabolites, including lignins, lignans, salicylates, coumarins, stilbens, styrylpyrones and all compounds embraced by the common name flavonoids – such as chalcones, isoflavonoids, flavones, flavonoles, anthocyanins, condensed tannins, phlobaphenes and many others (Fig. 2.1.; Buchanan *et al*., 2002). The most abundant phenylpropanoid derivative is lignin (66.0% of all compounds synthesised in the phenylpropanoid pathway), followed by condensed tannins (18.6%) (Aksamit-Stachurska *et al*., 2008; Winkel-Shirley, 2001). The compounds emerging from this pathway have a wide variety of functions as structural, signalling and protective molecules. For this reason the pathway is the best-studied secondary metabolic pathway of all (Yu and McGonigle, 2005).

The precursors of the phenylpropanoid pathway are phosphoenolpyruvate (from glycolysis) and erythrose 4-phosphate (from the pentose phosphate pathway), leading to two important intermediates, shikimic and chorismic acid. The phenylpropanoid pathway itself begins with the action of phenyl ammonium lyase (PAL), which deaminates the amino-acid phenylalanine derived from chorismate. The cinnamic acid so formed is then converted, in several steps, into chalcon. The biosynthetic pathway leads from chalcon to flavanones and then to isoflavones, which are the subject of our interest. The other main biosynthetic steps and branches of the pathway are shown in the diagram below (according to Buchanan *et al*., 2002; Fig. 2.1.).

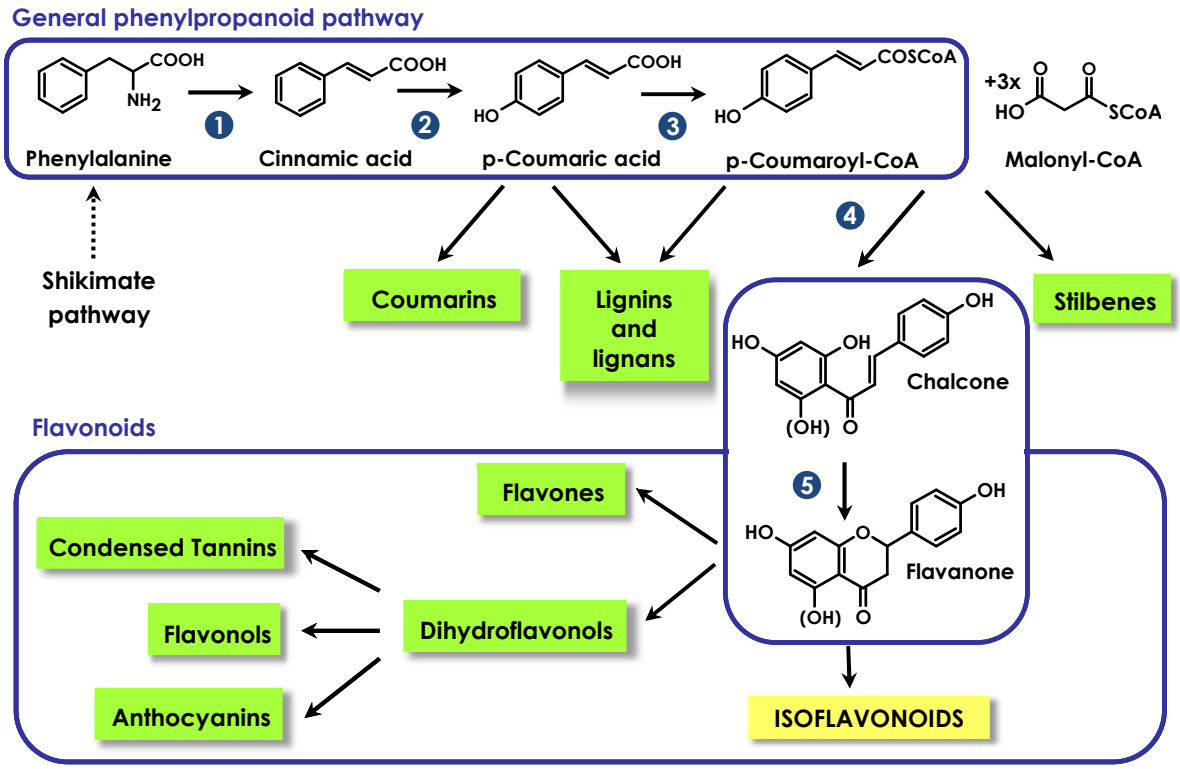


Fig. 2.1. Schematic of the major branches of the phenylpropanoid pathway. Enzymes involved: (1) phenylamonium lyase (PAL), (2) cinnamate-4-hydroxylase (C4H), (3) 4-coumaroyl:CoA-ligase (4CL), (4) chalcone synthase (CHS), (5) chalcone isomerase (CHI). Drawn in ACD/Chemsketch after Buchanan *et al*., 2002 and Winkel-Shirley, 2001.

2.1.2. The biosynthetic pathway leading to the production of isoflavonoids

The flavonoid – and thus isoflavonoid – pathway starts with the condensation of p-coumaroyl-CoA with three molecules of malonyl-CoA, to form the C15 flavonoid skeleton – **chalcone.** This can be the tetrahydroxychalcon named naringenin-chalcon (formed by the catalysis of chalcone synthase), or the trihydroxychalcon named isoliquiritigenin (formed through the catalysis of chalcone synthase and chalcone reductase). From these structures arise the ubiquitous flavanone **naringenin,** and the less abundant flavanone **liquiritigenin,** respectively (both by chalcone isomerase catalysis). Interestingly, chalcone isomerase from non-leguminous species is unable to catalyse the isomerization of isoliquiritigenin into liquiritigenin (Shimada *et al*., 2003).

The aforementioned flavanones are direct precursors of the isoflavones **genistein** and **daidzein**, respectively, which are the first isoflavonoids to appear in the pathway (Fig. 2.2.). The conversion of the flavanones to isoflavones is catalysed by the unusual

action of isoflavone synthase (2-hydroxyisoflavanone synthase, detailed description in the section 2.2.) The flavanone naringenin, however, is utilized by several other enzymes from the flavonoid metabolism (e.g. flavanone-3-hydroxylase, flavone synthase etc.), thus competing with IFS for the substrate (Yu and McGonigle, 2005).

The further metabolism of isoflavones involves enzymes such as glycosyltransferases, prenyltransferases, methyltrasferases and others, all of which are characterized by a wide substrate specificity (Lapčík, 2007). Their mode of action could partly explain the phenomenon "Too few genes, too many metabolites" (Schwab, 2003), reflecting the considerably higher number of metabolites compared to the limited number of known genes encoding for the discussed enzymes.

According to Veitch (2007), one of the essential processes leading to isoflavonoid diversification is the hydroxylation of the C-2´and C-3´positions by the isoflavones 2´-hydroxylase and 3´-hydroxylase, respectively. Another important and well-defined modification is the methylation of daidzein and its precursor 2,7,4´-trihydroxyisoflavanone, resulting in the production of the isoflavones isoformononetin and formonetin, respectively (Akashi *et al*., 2000). The latter is a key precursor of isoflavans, coumestans and pterocarpan phytoalexins (e.g. medicarpin or pisatin; Fig. 2.2.).

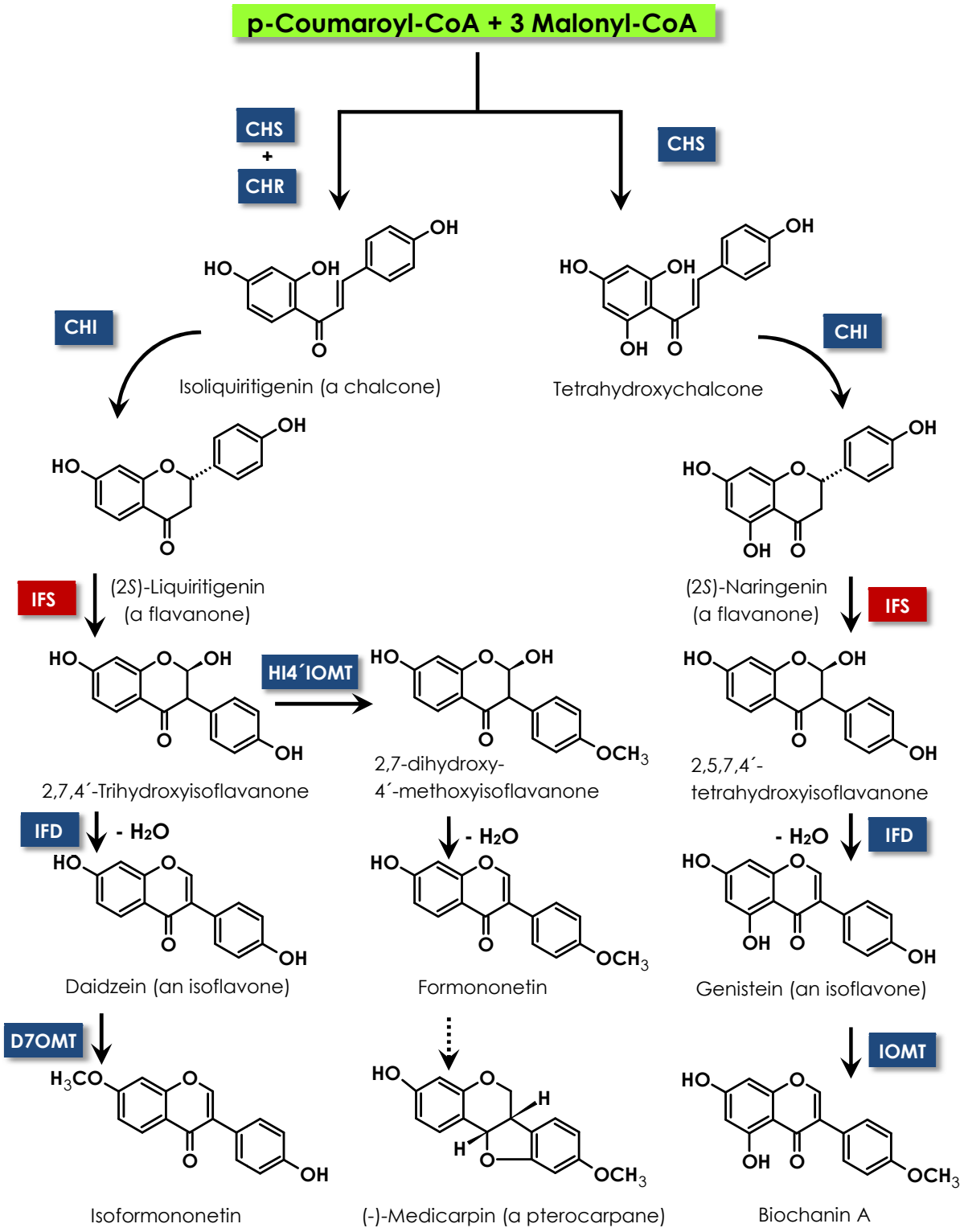


Fig. 2.2. Schematic of the isoflavonoid biosynthetic pathway. Enzymes involved: **CHS**, chalcone synthase; **CHI**, chalcone isomerase; **IFS**, isoflavone synthase; **IFD**, isoflavone dehydratase; **IOMT**, isoflavanone *O*-methyltransferase; **HI4´IOMT**, hydroxyisoflavanone 4´-*O*-methyltransferase; **D7OMT**, daidzein 7-*O*-methyltransferase. Drawn in ACD/ChemSketch after Buchanan *et al*., 2002 and Veitch, 2007.

2.2. Isoflavone synthase (IFS)

Isoflavone synthase, IFS (also called 2-hydroxyisoflavanone synthase, 2-HIS) plays a key role in the biosynthesis of the plant secondary metabolites, isoflavonoids. This enzyme was discovered as a cytochrome P450 monooxygenase by the Griesebach group at the University of Freiburg in 1984 (Hagmann and Griesebach, 1984). This was when it was first proclaimed: "The 'isoflavone synthase' was found in elicitor-challenged soybean cell cultures". They reported that microsomes prepared from elicitor-challenged soybean cell cultures catalysed the NADPH-dependent and dioxygen-dependent rearrangement of radio-labelled naringenin to genistein. Many years later, IFS was classified as a member of the subfamily CYP93C. It will be treated in greater detail below.

2.2.1. Cytochromes P450 (CYPs)

Plant P450 monooxygenases are membrane-bound proteins consisting of a heme and of an apoprotein that is responsible for substrate specificity (Bolwell *et al*., 1994). Utilizing reducing equivalents from the NADPH cofactor, they catalyse an enormous range of oxidative reactions across all kingdoms. In plants they are involved in the metabolism of lipids, phenylpropanoids, alkaloids, terpenoids and other secondary metabolites (Ayabe and Akashi, 2006).

The heme prosthetic group is bound through a cystein residue in a highly-conserved domain near the C-terminus (Bolwell *et al*. 1994), in a position nearly parallel to the surfaces between the L and I helices. Although the CYPs consist of conserved α-helices and β-sheets, they display narrow substrate specificity, as well as strict regio- and stereo-specificities (Mansuy, 1998).

Several X-ray structures of some vertebrate and bacterial CYPs have been described. According to the available literature, however, plant cytochromes P450 have not yet been obtained and thus the question of the precise structure of these proteins remains to be solved.

2.2.2. CYP93C

In 1999-2000, that is as late as 16 years after its discovery, IFS was confirmed to be the cytochrome P450 on the basis of genomic studies carried out independently by three research groups (Steele *et al*. 1999; Akashi *et al*. 1999; Jung *et al*., 2000).

Based on the sequence homology and P450 nomenclature, all isoflavone synthases of which the genes had been cloned were placed in the subfamily CYP93C, and denoted by Dr. David Nelson (2009) by list numbers (Tab. 2.1.).

The sequences evince the typical features of cytochromes P450, including "A"-"L" α-helices (the "I" helix is oxygen-binding), heme-binding motifs near the C-terminus, and conserved PERF domain (Steele *et al*., 1999; P450 Engineering Database, University of Stuttgart, http://www.cyped.uni-stuttgart.de/).

To date (as of July 2010), 28 individual IFS-amino-acid sequences (including IFS isoforms), displaying a homology of more than 95%, have been described in a total of 17 leguminous species, according to the P450 Engineering Database. Moreover, three recent additions have appeared in the GenBank: two IFS isoforms from the legume *Lupinus luteus* (Madrzak and Narozna, 2008, unpublished) and one IFS sequence from the legume *Cullen corylifolium* (Misra *et al.*, 2010). The presence of IFS in non-leguminous species has hitherto been reported just once – in the single case of *Beta vulgaris*, from the family Chenopodiaceae, where two *IFS* isoforms have been found (Jung *et al*., 2000).

The *IFS* gene from *Glycine max* (soybean) was first described more or less at the same time independently by Steele *et al*. (1999) and by Jung *et al*. (2000). Jung *et al*. isolated two IFS isoforms (IFS1 and IFS2) sharing highly homologous regions (92.5% at the nucleotide level and 96.7% at the amino acid level). Both isoforms can convert the flavanone substrates to isoflavones, but with differing degrees of efficiency (Jung *et al*., 2000). Since that time, the gene has been successfully over-expressed in several non-leguminous species that do not produce isoflavonoids, namely *Arabidopsis thaliana* (Jung *et al*., 2000 and others), *Nicotiana tabacum* (Jung *et al*., Yu *et al.*, 2000, Liu *et al*., 2007), *Solanum lycopersicum* (Shih *et al*., 2008), *Petunia hybrida Vilm*. and *Lactuca sativa* (Liu *et al*., 2007), *Oriza sativa* (Sreevidya *et al.*, 2006), and *Zea mays* black mexican sweet cells (Yu *et al*., 2000). These studies were motivated by potential applications in the metabolic engineering of isoflavonoids, but the yield of isoflavonoids was several orders of magnitude lower than in soybean seeds (which contain approximately 200 mg isoflavonoids per 100 g of beans; Mazur and Adlercreutz, 1998).

P450	Species	GenBank number	Reference
CYP93C1	*Glycine max*	AF022462	Siminszky *et al*., 1999
CYP93C1v2	*Glycine max*	AF135484	Steele *et al*, 1999
CYP93C2	*Glycyrrhiza echinata*	AB023636	Akashi *et al*., 1999
CYP93C3	*Cicer arietinum*	AF243804	Overcamp *et al*., 2000
CYP93C4	*Glycine max*	AF089850	Wu and Verma, dir.sub.1998
CYP93C5	*Glycine max*	AF195818	Jung *et al*., 2000
CYP93C6v1	*Vigna radiata*	AF195806	Jung *et al*., 2000
CYP93C6v2	*Vigna radiata*	AF195807	Jung *et al*., 2000
CYP93C6v3	*Vigna radiata*	AF195808	Jung *et al*., 2000
CYP93C6v4	*Vigna radiata*	AF195809	Jung *et al*., 2000
CYP93C7v1	*Medicago sativa*	AF195801	Jung *et al*., 2000
CYP93C7v2	*Medicago sativa*	AF195802	Jung *et al*., 2000
CYP93C8	*Medicago sativa*	AF195800	Jung *et al*., 2000
CYP93C9v1	*Trifolium pratense*	AF195810	Jung *et al*., 2000
CYP93C9v2	*Trifolium pratense*	AF195811	Jung *et al*., 2000
CYP93C10v1	*Trifolium repens*	AF195814	Jung *et al*., 2000
CYP93C10v2	*Trifolium repens*	AF195815	Jung *et al*., 2000
CYP93C11v1	*Beta vulgaris*	AF195817	Jung *et al*., 2000
CYP93C11v2	*Beta vulgaris*	AF195816	Jung *et al*., 2000
CYP93C12	*Lens culinaris*	AF195805	Jung *et al*., 2000
CYP93C13	*Lens culinaris*	AF195804	Jung *et al*., 2000
CYP93C14	*Pisum sativum*	AF195812	Jung *et al*., 2000
CYP93C15	*Vicia villosa*	AF195803	Jung *et al*., 2000
CYP93C16	*Lupinus albus*	AF195813	Jung *et al*., 2000
CYP93C17	*Lotus japonicus*	AB024931	Shimada *et al*., 2000
CYP93C18	*Pisum sativum*	AF532999	Cooper *et al*., 2005
CYP93C19	*Medicago truncatula*	AY167424	Butelli *et al*., dir .sub. 2002
CYP93C20	*Medicago truncatula*	AF195812	Deavours and Dixon, 2005
CYP93C21	*Pueraria montana var. lobata*	AF462633	Jeon and Kim, dir.sub. 2001
CYP93C22	*Astragalus membranaceus*	DQ205408 (partial IFS)	Pan *et al*., dir sub. 2005
	Glycine soja	EU391469 (*IFS*1) EU391516 (*IFS*2)	Chenk *et al*., dir.sub. 2008
	Vigna unguiculata	EU616497 (*IFS*1) EU616500 (*IFS*2)	Kaur and Murphy, dir.sub. 2008
	Lupinus luteus	FJ539089 (*IFS*1) FJ539090 (*IFS*2)	Madrzak and Narozna, dir.sub. 2009
	Cullen corylifolium	GU322814	Mistra *et al*., 2010

Tab. 2.1. List of cloned *IFS* genes from GenBank (http://www.ncbi.nlm.nih.gov/nucleotide/), P450 Database (http://drnelson.uthsc.edu/cytochromeP450.html) and from the P450 Engineering Database, University of Stuttgart (http://www.cyped.uni-stuttgart.de/). After Yu and McGonigle, 2005 (updated). Abbreviation: dir. sub – direct submission.

2.2.3. Reaction mechanism of IFS

In spite of the fact that the conversion of flavanones into isoflavones is a two-step process, the overall reaction is frequently ascribed to IFS (Veitch, 2007). In fact, due to the action of membrane-bound IFS, an unstable intermediate 2-hydroxyisoflavanone arises from a flavanone precursor (Fig 2.3.). For this reason, "2-hydroxyisoflavanone synthase" or "2-HIS" is a more precise name for the enzyme involved (Yu and McGonigle, 2005). The subsequent step is catalyzed by a soluble 2-hydroxyisoflavanone dehydratase, which is responsible for the 1,2-elimination of water from 2-hydroxyisoflavanones and the formation of a double bond between the C2 and C3 positions of the resulting isoflavone structures (Akashi *et al*., 2005; Fig 2.3.).

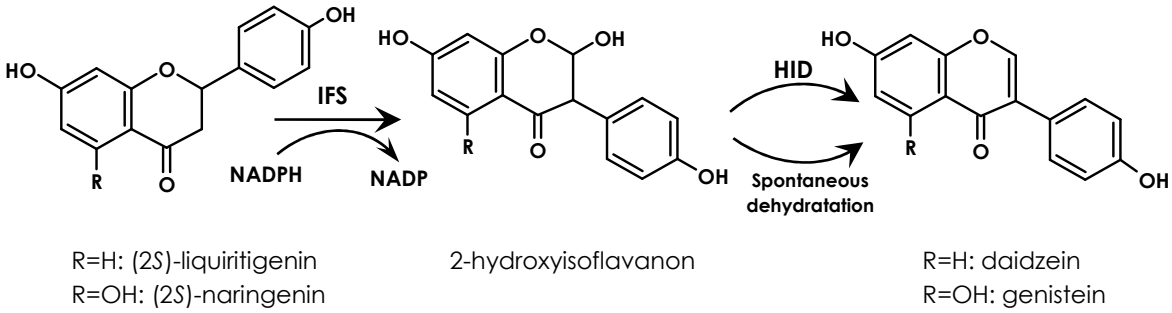


Fig. 2.3. The biosynthesis of isoflavone from flavanone, in detail. **IFS**, isoflavone synthase; **HID**, 2-hydroxyisoflavanone dehydratase. Drawn in ACD/ChemSketch, after Tian and Dixon, 2006.

Yu and McGonigle, who were amongst the first discoverers of the isoflavone synthase gene, called IFS an "intriguing enzyme" because of its outstanding ability to catalyze both the hydroxylation of the two flavanone precursors – liquiritigenin and naringenin – and the critical migration of the aryl group from the C2 to the C3 position on the chromene skeleton of the aforementioned flavanones (Yu and McGonigle 2005).

The reaction mechanism of the 1,2-aryl migration was studied in elicitor-challenged *Pueraria lobata* cell cultures (Hashim *et al*., 1990; Hakamatsuka *et al*., 1991). The authors suggested a new reaction mechanism which, in contrast to the previous concept of enol-epoxidation of the flavanone (Kochs and Grisebach, 1986), involved hydroxylation associated with the rearrangement of the flavanone (Fig. 2.4.). The reaction requires interaction with NADPH:cytochrome P450 reductase, which provides the P450 with electrons by reducing the NADPH. The mechanism involves (1)

the abstraction of hydrogen from C3 by an activated oxygen intermediate, bound to heme iron, followed by (2) the migration of the aryl group from position C2 to C3 on the chromene skeleton, and (3) the rebinding of the hydroxyl radical to C2 (Sawada *et al*., 2002). It is not clear whether or not IFS assists the dehydratase in the subsequent step – the elimination of water (Yu and McGonigle, 2005). Although the reaction catalysed by IFS is frequently called a "unique" reaction, it cannot be excluded that examples of a similar reaction mechanism might be found within the complex plant secondary metabolism.

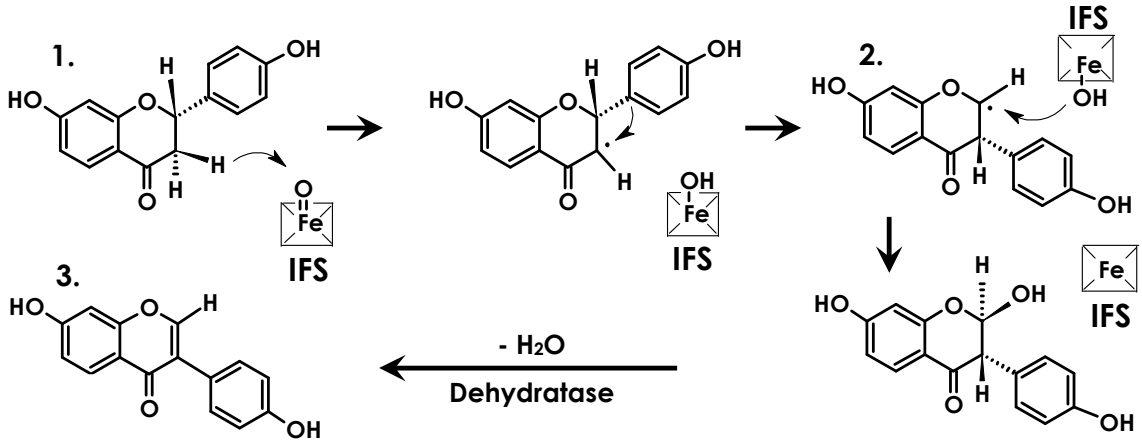


Fig. 2.4. The probable reaction mechanism of oxidative aryl migration, catalyzed by isoflavone synthase (IFS) and leading to the production of isoflavones. (1) liquiritigenin, (2) 2,7,4´-trihydroxyisoflavanone (2-hydroxyisoflavanone, for short), (3) daidzein. Drawn in ACD/ChemSketch after Hakamatsuka *et al*., 1991.

2.2.4. Molecular modelling of the IFS active site

In 2002, Sawada and his colleagues reported the identification of two amino-acid residues that are critical for aryl migration (Sawada *et al*., 2002). On the basis of the known X-ray crystal structure of the cytochrome P450BM3 from *Bacillus megaterium* (Ravichandran *et al*., 1993), and using a multiple-alignment analysis with members of the CYP93 family, they generated a 3-D model of CYP93C2 (IFS from *Glycirrhiza echinata*; Fig. 2.5.). Several potentially crucial amino-acids were marked out by docking (2*S*)-liquiritigenin into the modelled IFS active site. Point-mutated proteins were then expressed in a heterologous yeast system, with the following results: (1) the wild-type IFS produced 2-hydroxyisoflavanone and a reaction by-product, 3-hydroxyflavanone, in a ratio of 92:8; (2) the protein with Ser 310→Thr mutation (centre

of I helix) yielded 2-hydroxyisoflavanone and by-products, in ratio of 57:43, indicating an increased proportion of by-products; and (3) the mutant protein with Lys 375→Thr produced only 3-hydroxyflavanone. Clearly, Lys 375 of CYP93C2 is essential for aryl migration, whilst the role of Ser 310 is more equivocal. The ε-amino group of Lys 375 probably acts as an anchor for the substrate, as it is proximal to substrate hydroxyl at C7. The probable role of Ser 310 is to facilitate the aryl migration, by providing the reaction with more space than Thr (Thr is placed at the position 310 in the case of all CYP93s with the exception of the subfamily CYP93C).

In a further study, Sawada and Ayabe (2005) introduced Leu 371 as an additional key amino-acid residue of IFS. Leu 371 is located near the substrate and appears to control the substrate accommodation in the active site and to contribute to the correct P450 fold.

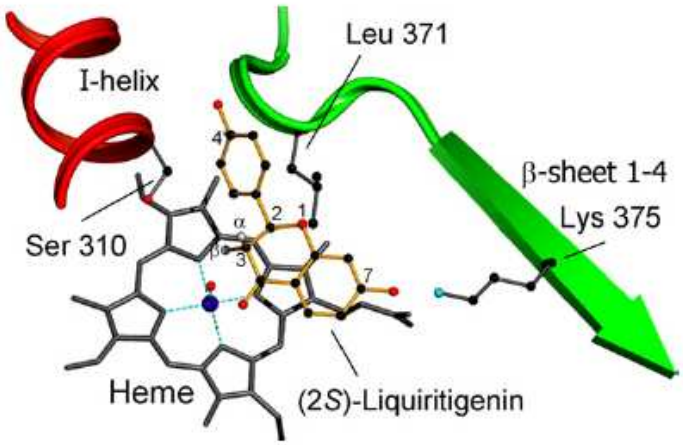


Fig. 2.5. *In silico*-generated model of the active site of IFS (CYP93C2), including accommodated liquiritigenin (in yellow; adapted from Sawada and Ayabe, 2005).

2.3. 2-hydroxyisoflavanone dehydratase (2-HID)

2-hydroxyisoflavanone dehydratase (2-HID or IFD), together with isoflavone synthase (IFS), is responsible for the formation of the isoflavonoid skeleton. 2-HID was first purified and characterized by Hakamatsuka *et al*. from yeast extract-elicited cell suspension cultures of *Pueraria lobata* (Hakamatsuka *et al.*, 1998). It is a single polypeptide with a molecular weight of 38 kDa, isoelectric point at pH 5.1 and a pH optimum at 6.8. It requires no co-factor.

Akashi *et al*. (2005) further reported cloning 2-HID cDNA from *Glycine max* and *Glycirrhiza echinata*. The amino-acid sequences of both 2-HIDs possesed the motifs characteristic to carboxylesterases, which had never been considered to exhibit dehydratase activity (Akashi *et al*., 2005). In other legumes, the production of isoflavones from 2-hydroxyisoflavones is most probably catalyzed by 2-HID homologues. However, in the case of non-leguminous plants the possible presence of a general "flavonoid dehydratase", or the spontaneous dehydratation of the intermediate, is more probable (Yu and McGonigle, 2005).

2.4. Isoflavonoids

2.4.1. Structure and classification of isoflavonoids

Isoflavonoids constitute a sub-class of the phenolic secondary metabolites – flavonoids, and thus have the same 15-carbon-skeleton of C6-C3-C6. In contrast to the flavone, the B-ring of the basic structure of isoflavone is attached at the C-3 rather than C-2 position (Crozier *et al*., 2006; Fig. 2.6., in which the biosynthetic origin of the skeleton is highlighted).

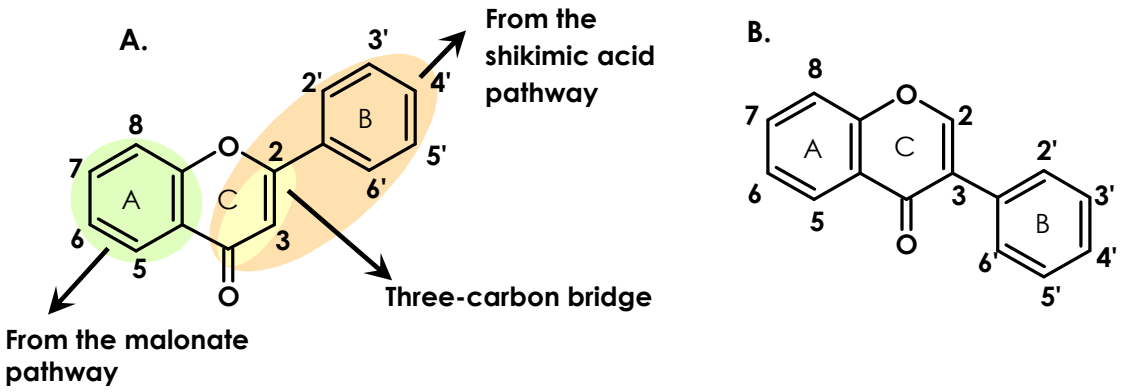
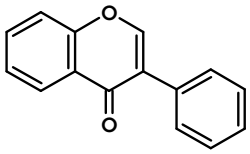
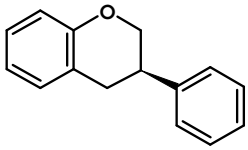
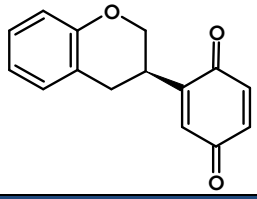
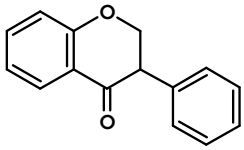
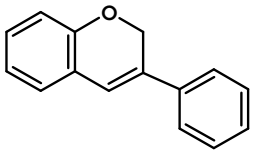
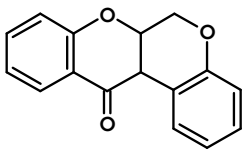
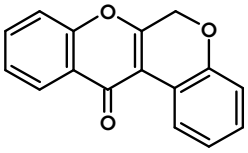
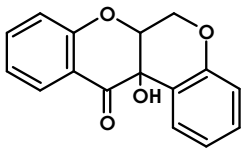
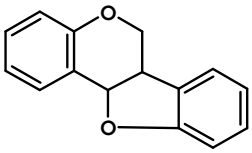
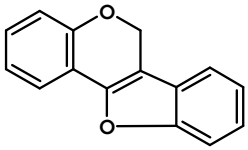
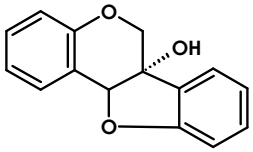
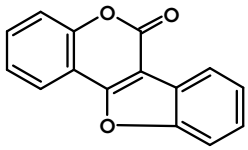
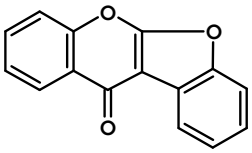
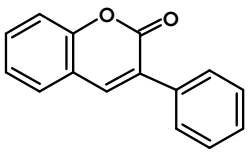
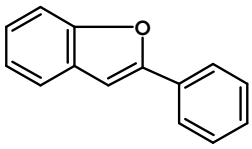
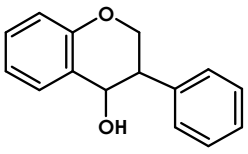


Fig. 2.6. The basic backbone of (A) flavone (2-phenylchromen-4-one; its biosynthetic origin shaded) and (B) isoflavone (3-phenylchromen-4-one). Drawn in ACD/ChemSketch after Crozier *et al*., 2006)

Isoflavonoids undergo various modifying reactions, such as hydroxylation, methylation, prenylation, chlorination, the addition of aromatic or aliphatic acids and extra heterocyclic rings, cyclization, dimerization, etc. (Reynaud *et al*., 2005).

In plants, isoflavonoids occur as free aglycones or they accumulate in the form of glycoconjugates, with glucose as the most common sugar component (Reynaud *et al*.,

2005). The conjugation step confers stability and solubility to the isoflavone aglycones, enabling their compartmentalization to vacuoles or their transportation to the site of accumulation. Glycosides are further converted into their respective malonyl derivatives (Dhaubhadel *et al*., 2008). This process enhances their solubility, protects glycosides from degradation by glycosidases and helps in their intracellular transport (Dhaubhaudel *et al*., 2008). The various modifications of isoflavonoids account for the multiplicity of their subgroups. According to Veitch (2007), sixteen different isoflavonoid subclasses can be distinguished, as shown in Tab. 2.2.

Isoflavones	Isoflavans	Isoflavanquinones	Isoflavanones
			
Isoflav-3-enes	**Rotenoids**	**Dehydrorotenoids**	**12a-Hydroxyrotenoids**
			
Pterocarpans	**Pterocarpenes**	**6a- Hydroxypterocarpans**	**Coumestans**
			
Coumaronochromones	**3-Arylcoumarins**	**2-Arylbenzofurans**	**Isoflavanols**
			

Tab. 2.2. Overview of the individual subgroups of isoflavonoids and their basic chemical structures. Drawn in ACD/ChemSketch after Veitch, 2007.

2.4.2. Isolation and identification of isoflavonoids

Isoflavonoids can be isolated from fresh, dried or lyophilised plant material. They occur more often in roots, rhizomes, wood, bark, shoots and seeds, than in leaves or flowers (Dacora and Phillips, 1996; Reynaud *et al*., 2005). However, depending on both biotic and abiotic factors, isoflavonoids can accumulate in any part of the plant (Dacora and Phillips, 1996).

As far as analytical techniques are concerned, the combination of high-performance liquid chromatography (HPLC) with mass spectrometric detection (MS), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), has proved to be a useful tool (Boland and Donnelly, 1998). Gas chromatography, nuclear magnetic resonance and capillary electrophoresis are also commonly employed. Apart from these, immunochemical methods such as fluorescence immunoassay (FIA), radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) have also been found to be very sensitive and effective (Lapčík *et al*., 1999; Lapčík *et al*., 2004).

The constantly growing sensitivity of methods for the analysis of natural products now enables the detection of isoflavonoids even in such plants where only traces can be found or in which the occurence of isoflavonoids had not been expected.

2.4.3. Taxonomical distribution of isoflavonoids

In the middle of the 19th century, Reinsch and Hlasiwetz first discovered the isoflavonoid ononin in the roots of the legume *Ononis spinoza* L. Surprisingly, further isoflavonoids were reported in connection with non-leguminous species, namely iridin from *Iris florentina* (Iridaceae; de Laire and Tiemann, 1893) and prunetin from *Prunus* sp. (Rosaceae; Finnemore, 1910) (cited from Veitch, 2007 and Lapčík, 2007).

Due to their ubiquitousness in the Fabaceae family, isoflavonoids have been considered as chemosystematic markers for this taxon in the past (Reynaud *et al*., 2005). However, the presence of isoflavonoids from sources other than legumes was also reported. Three successive reviews (Reynaud *et al*., 2005; Macková *et al*., 2006; Lapčík, 2007) attempted to summarize the current knowledge of the distribution of isoflavonoids in non-leguminous families. According to Lapčík and Veitch, at least 225 (predominantly isoflavones) out of over 1600 known isoflavonoids have been described in 59 non-leguminous families (Lapčík, 2007; Veitch, 2007). By the year 2007, isoflavonoids had been found in one family of mosses (Bryopsida), three families of

conifers (Pinopsida), 10 families of monocots (Liliopsida) and 46 families of dicots (Magnoliopsida; Macková *et al*., 2006; Lapčík, 2007; Tab. 2.3.).

Thus, small amounts of isoflavonoids are also present in our daily diet, in foodstuffs of plant origin. In addition to seeds and other parts of leguminous plants (especially all soya products), these compounds have also been found in small amounts in certain fruits, vegetables, cereals, potatoes, oil seeds and nuts (Boker *et al*., 2002, Mazur and Adlercreutz, 1998). Isoflavonoids were also detected in green tea and coffee (Mazur *et al*., 1998), beer (Rosenblum *et al*., 1992) and even bourbon (Gavaler *et al*., 1987).

The effects of dietary isoflavonoids, including potential benefits and risks to human health, will be discussed below.

Division	Family	Division	Family		
Bryopsida	Bryaceae	**Magnoliopsida**	Amaranthaceae	Cucurbitaceae	Polygalaceae
Pinopsida	Araucariaceae		Apiaceae	Erythroxylaceae	Polygonaceae
	Cupressaceae		Apocynaceae	Euphorbiaceae	Rhamnaceae
	Podocarpaceae		Asclepiadaceae	Magnoliaceae	Rosaceae
Liliopsida	Asphodelaceae		Asteraceae	Malvaceae	Rutaceae
	Cyperaceae		Bombacaceae	Melastomataceae	Rubiaceae
	Eriocaulaceae		Brassicaceae	Menispermaceae	Sapotaceae
	Iridaceae		Cannabaceae	Moraceae	Scrophulariaceae
	Juncaceae		Caryophyllaceae	Myricaceae	Solanaceae
	Liliaceae		Celastraceae	Myristicaceae	Sterculiaceae
	Poaceae		Chenopodiaceae	Myrtaceae	Urticaceae
	Smilaceae		Clusiaceae	Nymphaeaceae	Verbenaceae
	Stemonaceae		Connaraceae	Nyctagicaeae	Vitaceae
	Zingiberaceae		Convolvulaceae	Ochnaceae	Violaceae
			Crassulaceae	Papaveraceae	Zygophyllaceae

Note: Isoflavonoids appear to have been detected in some additional families (Lauraceae, Pedaliaceae etc.), but the evidence has not been considered to be reliable (Macková *et al*., 2006).

Tab. 2.3. Overview of non-leguminous taxa producing isoflavonoids, as of 2007 (after Reynaud *et al*., 2005; Macková *et al*., 2006; Lapčík, 2007).

2.4.4. Biological functions of isoflavonoids in plants

The role played by isoflavonoids in plant defence and in the induction of rhizobial symbiosis is frequently discussed in the literature.

Due to their antimicrobial activity, some isoflavonoids are classified as phytoalexins (i.e. antimicrobial compounds synthetised *de novo* in direct response to

microbial attack and other stressors) and as phytoanticipins (i.e. molecules chemically identical to phytoalexins but stored in plant cells in anticipation of pathogenic attack or produced after infection solely from preexisting constituents) (VanEtten *et al*., 1994). Apart from isoflavonoids, which are the most widely studied class of phytoalexins, such defensive compounds can be found among terpenes, stilbene, benzofuranes and other chemical groups (Dakora and Phillips, 1996).

Amongst the most potent phytoalexins in leguminous plants are the isoflavanone kieviton (*Phaseolus, Vigna*); pterocarpans phaseollins (*Phaseolus*), pisatin (*Pisum*), medicarpin (*Cicer, Medicago*), glyceollins (*Glycine*), maackiain (*Trifolium*); and the coumestan (a fully-oxidised pterocarpan) coumestrol (*Glycine, Medicago, Phaseolus, Vigna*). However, the simple isoflavones daidzein, genistein, formononetin, glycitein and biochanin A also display some degree of antimicrobial and antifungal activity (Dakora and Phillips, 1996). A few examples of isoflavonoid phytoalexins have been detected also in several non-leguminous families (Chenopodiaceae, Myristicaceae, Zingiberaceae, Iridaceae and others; Reynaud, 2005 and Lapčík, 2007). Moreover, rotenoids (especially rotenone) from both leguminous and non-leguminous species are known for their insecticidal, piscicidal and antiviral activities (Boland and Donnelly, 1998).

The production of isoflavonoid phytoalexins is regulated at the level of transcription of the genes which are required for the isoflavonoid biosynthesis. Biotic elicitors (e.g. saccharides from the cell wall of the plant or of the pathogen) as well as abiotic factors (e.g. UV radiation, heavy metals, too low or too high concentrations of minerals, etc.) play an important role in the stimulation of isoflavonoid formation. The elicited isoflavonoids can then act as toxins against the pathogens; but the signalling function of isoflavonoids in connection with hypersensitive response (HSR) and systemic acquired resistance (SAR), still awaits clarification (Dacora and Phillips, 2006).

The concept of the role of isoflavonoids as phytoalexins is somewhat complicated by the fact that some isoflavonoids function as stimulants of the mutualistic interactions between leguminous plants and the soil diazotropic bacteria collectively called rhizobia. Chemoattractants – isoflavonoids and flavonoids – are excreted from roots and interact with rhizobial NodD proteins, which serve as both environmental sensors and activators of the transcription of rhizobial *nod* genes. This induction leads to the synthesis of chitolipooligosaccharidic Nod factors which, in turn, through positive

feedback, stimulate the further production of (iso)flavonoids, provoke the nodulation of root hairs and allow rhizobia to enter the root through the infection thread (Broughton *et al*., 2000; Cooper, 2004; Fisher and Long, 1993). Successful infection eventually results in the reduction of atmospheric N_2 to ammonia (a form of nitrogen acceptable for plants) by rhizobial enzyme nitrogenase.

Subramanian *et al.* (2004) showed that the expression of soybean IFS increased in root hairs as well as in xylem, in response to *Bradyrhizobium japonicum* treatment. *IFS* silencing using RNA interference resulted in a reduction of isoflavone levels, as well as of nodulation efficiency in soybean hairy roots; and even feeding the IFS RNAi roots with the isoflavone genistein was not sufficient to restore the nodulation (Subramanian *et al*., 2006). Moreover, it was found that the IFS RNAi roots had a significantly higher level of auxin transport (the ability of the isoflavone genistein to act as auxin transport inhibitor was demonstrated long ago; Jacobs and Rubery, 1988). According to these findings, isoflavonoids play a critical role as *nod* gene inducers inside the root. Also, there is a clear connection between isoflavonoid accumulation and the modulation of auxin transport, although this connection was found not to be essential for nodulation (Subramanian *et al*., 2007). Subramanian *et al.* also cloned and characterized the promoters of soybean, IFS1 and IFS2, and found them to be root- and seed-specific. These two promoters respond differently to stimuli such as defence or nodulation signals (Subramanian *et al*., 2004).

Another interesting result was obtained by Sreevidya *et al*., who introduced the soybean *IFS* gene into rice (Sreevidya *et al*., 2006), which resulted in the transgenic plants producing genistein in glycoside form. In addition, these plants were able to induce *nod* gene expression, as demonstrated by experiments with different strains of rhizobia (Sreevidya *et al*., 2006).

2.4.5. Pharmacological effects of isoflavonoids

In the last 20 years, isoflavonoids have attracted much attention due to their multiple beneficial effects to human health. Isoflavones genistein and daidzein, the richest source of which is dietary soybean (*Glycine max* (L.) Merr., Fabaceae), are known as effective phytoestrogens. There is a countless number of publications dealing with this phenomenon.

In brief, isoflavones have a structure which enables them to mimic the endogenous hormone estradiol and to bind the estrogen receptors α and β (Ososki and Kennelly, 2003; Fig. 2.7.). They can act as both agonists and antagonists, i.e. they can respectively activate and block the signalling pathway leading to the expression of estrogen-responsive genes. Hence they are termed "selective estrogen receptor modulators" (SERMs) and are used as an alternative to hormone replacement therapy (HRT; Brzezinski and Debi, 1999). Apart from this, isoflavones have several non-genomic effects on humans, such as, for example, the inhibition of aromatase, tyrosine kinase and DNA topoisomerase (Kurzer and Xu, 1997), or interactions with sex hormone-binding globulins (Mousavi and Adlercreutz, 1993).

Undoubted health benefits resulting from these and other molecular effects include the decreased incidence of certain types of cancer, reduced (post-)menopausal symptoms, the prevention of osteoporosis and of cardiovascular diseases, antioxidant action and many others (Corwell *et al*., 2004).

However, the potential risks connected with isoflavonoid consumption are also frequently discussed in the literature. One such adverse effect was described as early as in 1946, when it was first established that sheep grazing on clover pastures with high levels of the isoflavone formononetin suffered from multiple fertility problems (Bennets *et al*., 1946). Some studies with rodents or even clinical studies with women demonstrated, both the stimulatory and inhibitory effects of isoflavones on breast cancer cell growth (Duffy *et al*., 2007). It is obvious that additional reasearch is required and that all results must be interpreted with a fair amount of caution.

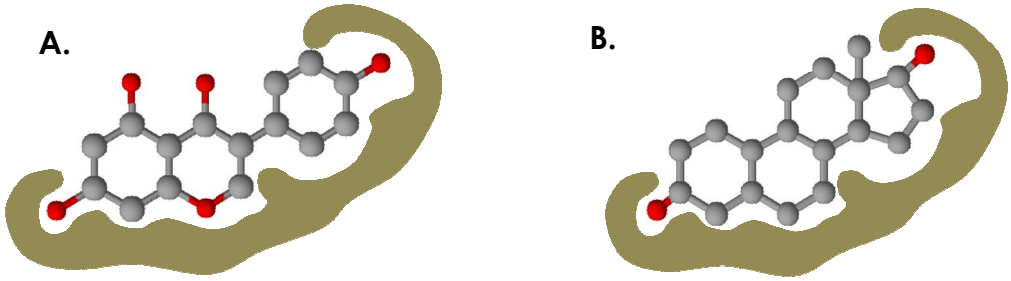


Fig. 2.7. Comparison of genistein (A) and estradiol (B) structures when bound to estrogen receptor. Drawn in ACD/ChemSketch after Demmig-Adams a McCauley, 2005.

2.5. Metabolic engineering of isoflavonoid biosynthesis

Since the flavonoid and isoflavonoid pathways are well-characterized pathways of the secondary metabolism in plants, they present an excellent target for metabolic engineering (Dixon and Steele, 1999). Moreover, isoflavonoids have been shown to play an important role in plant defence, symbiotic interactions as well as in human health. The genetic manipulation in plants – particularly crops – which do not naturally produce isoflavonoids should thus provide the plants with several beneficial functions. On the other hand, it would be desirable to produce genetically-modified isoflavone-deficient plants as fodder for grazing animals (Crozier *et al*., 2006).

The introduction of the isoflavonoid pathway into non-legumes can theoretically be achieved by transformation with a single enzyme – IFS (Liu *et al*., 2002). However, such attemps up to now have resulted in insufficient levels of isoflavonoids produced in transformed plants (Du *et al*., 2010). The bottleneck for engineered isoflavonoid production is the competition for flavanone substrate between IFS and different endogenous branches of the flavonoid pathway, especially common flavonol synthesis. Clear evidence for this was provided by Liu *et al*. (2002) who introduced IFS into the tt6/tt3(F3H/DFR) double mutant with blocked flavonol and anthocyanin biosynthesis. As a result, they achieved significantly higher levels of accumulated genistein.

The metabolic channelling through a metabolon (Fig. 2.8.) – for which a model was proposed by Liu and Dixon (2001) and Yu and McGonigle (2005) – can thus complicate possible engineering strategies, by limiting the access of introduced enzymes to their substrates (Dixon and Steele, 1999). The correct localization and transport of the introduced protein play an important role. In the case of membrane-bound IFS, the protein has to migrate from its site of synthesis and adopt the correct orientation for its accurate association with the ER membrane (Jaganath, 2005). A better understanding of how the metabolone is formed and knowledge of the interactions of the components as well as of the manner in which the pathways are regulated (the transcription factors specific to the isoflavonoid pathway have not been yet reported), can open the way to the successful metabolic engineering of isoflavonoids (Yu and McGonigle, 2005).

Although the present masters thesis is not concerned with the metabolic engineering of isoflavonoids, our methodical approach builds upon the same principles of genetic manipulation.

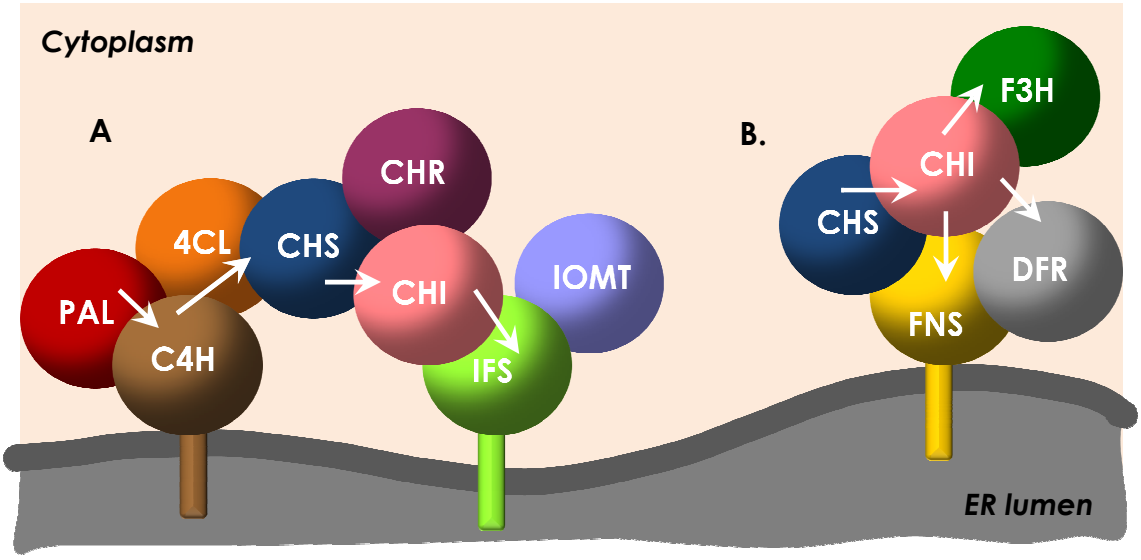


Fig. 2.8. Proposed isoflavonoid (A) and flavonoid (B) metabolons in the phenylpropanoid pathway. Arrowheads indicate metabolite flow. Enzymes involved: PAL, phenylalaninammonium lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; CHR, chalcone reductase; IFS, isoflavone synthase; IOMT, isoflavanone *O*-methyltransferase; FNS, flavone synthase; F3H, flavanone 3β-hydroxylase; DFR, dihydroflavonol 4-reductase. After Yu a McGonigle, 2005.

3. MATERIALS AND METHODS

3.1. Material

3.1.1. Plant material

Young leaves from both *Pisum sativum* L. and *Phaseolus vulgaris* L. were obtained from the greenhouses at the Czech University of Life Sciences in Prague and from a commercial source (Podravka-Lagris s.r.o., Czech Republic); young leaves from 33 species from 14 different non-leguminous families (Tab.1) were all obtained from the Czech University of Life Sciences in Prague, with the exception of *Cannabis sativa* (kindly provided by Dr. Martin Vágner, Institute of Experimental Botany AS CR) and *Beta vulgaris*, grown from seeds in room conditions (SEMO s.r.o, Czech Republic). Seeds from *Pachyrhizus tuberozus* (Lam.) Spreng. were imported from Peru (seeds provided by Wilfredo Guillen Huachua, Instituto Nacional de Investigación Agraria, Lima). *Arabidopsis thaliana* L. ecotype Col-0 and *Nicotiana benthamiana* Domin. were both provided by the Laboratory of pollen biology, Institute of Experimental Botany AS CR.

Family	Species	Family	Species
Annonaceae	*Annona cherimolia* *Annona muricata*	Lamiaceae (cont.)	*Thymus serpillum* *Thymus vulgaris*
Cannabaceae	*Cannabis sativa* *Humulus lupulus*	Lauraceae	*Persea americana*
		Pedaliaceae	*Sesamum indicum*
Ephedraceae	*Ephedra sinica*	Ranunculaceae	*Heleborus niger*
Erythroxylaceae	*Erythroxylon coca*	Rubiaceae	*Coffea arabica*
Chenopodiaceae	*Beta vulgaris*	Rutaceae	*Ruta graveolens*
Iridaceae	*Iris sp.*	Solanaceae	*Capsicum annuum* *Capsicum frutescens* *Nicotiana rustica* *Physalis peruviana* *Solanum lycopersicum* *Solanum muricatum* *Solanum melongena*
Lamiaceae	*Hyssopus officinalis* *Lamium album* *Levandula angustifolia* *Mentha piperita* *Ocimum basilicum* *Origanum vulgare* *Pogostemon cablin* *Rosmarinus officinalis* *Salvia officinalis* *Scutellaria baicaliensis*	Zygophyllaceae	*Tribulus terestris*
		Fabaceae (control)	*Phaseolus vulgaris* *Pachyrhizus erosus*

Tab. 3.1: Table of plant species selected for screening.

3.1.2. Bacterial strains and plasmids

- ***The competent cells used were:***

Escherichia coli – One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen,USA)

Alpha-Select Gold Efficiency Competent Cells (Bioline, UK)

Agrobacterium tumaefaciens strain GV3101

- ***The plasmids used were:***

pGEM®-T Easy (Promega, USA)

pCR8®/GW/TOPO® (Invitrogen, USA)

pENTR™/D-TOPO® (Invitrogen, USA)

pGWB2 and pGWB5 (obtained from Tsuyoshi Nakagawa, Shimane University, Matsue, Japan)

3.1.3. Antibiotics

Antibiotics	Final concentration	Selection marker for:
Ampicilin	**100 µg/ml**	pGEM®-T Easy in *E.coli*
Gentamycin	**50 µg/ml**	Ti-plasmid in *Agrobacterium*
Hygromycin	**50 µg/ml**	pGWB2 and pGWB5 in *Alpha, Agrobacterium and* in transformed *Arabidopsis*
Kanamycin	**50 µg/ml**	pENTR™/D-TOPO® in *E.coli*, pGWB2 and pGWB5 in *Alpha* and *Agrobacterium*
Spectinomycin	**100 µg/ml**	pCR®8/GW/TOPO® in *E.coli*
Rifampicin	**100 µg/ml**	Ti-plasmid in *Agrobacterium*

3.1.4. Growth media

- ***Bacterial growth media (w/v)***

S.O.C. medium (Invitrogen, USA)
LB Broth: 2.5% LB Broth; pH 7.2
YEB medium: 0.5 % peptone, 0.6 % yeast extract, 0.05% $MgSO_4.7H_2O$, 0.5% sucrose; pH 7.2
LB Agar: 3.5% LB Agar; pH 7.2
YEB Agar: YEB medium, 1.2 % Agar

- ***Plant growth media (w/v)***

½ MS medium: 0.2% Murashige and Skoog Basal Salt, 1% sucrose, 0.01% myo-inositol, 0.05 % MES, 0.8% agar; pH 5.7 + vitamins (1µg/ml of thiamin, 0.5 µg/ml pyridoxin and 0.5 µg/ml nicotine acid)

All media were autoclaved for 20 min at 121°C and at pressure 1kPa prior to the addition of appropriate antibiotics/vitamins.

3.1.5. Chemicals

Acetic acid	Lachner, Czech republic
Agar	Sigma-Aldrich, Germany
Agarose	Serva, Germany
Cetrimonium bromide (CTAB)	Sigma-Aldrich, Germany
5-Bromo-4-chloro-3-indolyl-β-D-galaktosid (X-Gal)	Duchefa Biochemie, Netherlands
5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP)	Sigma-Aldrich, Germany
Acetosyringone	Sigma-Aldrich, Germany
Ammonium acetate	Merck, Germany
Bovine Serum Albumine (BSA)	Sigma-Aldrich, Germany
Bromphenol blue	Serva, Germany
Dimethyl sulphoxide (DMSO)	Serva, Germany
Dimethylformamide (DMFO)	Sigma-Aldrich, Germany
Ethylenediaminetetraacetic acid (EDTA)	Serva, Germany
Ethidium bromide	Sigma-Aldrich, Germany
Ethyl Alcohol	Lachner, Czech republic
Glycerol	Sigma-Aldrich, Germany
Chloroform	Lachner, Czech republic
Isoamyl alcohol	Penta, Czech Republic
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, Germany
Isopropyl alcohol	Penta, Czech Republic
LB Agar	Duchefa Biochemie, Netherlands
LB Broth	Duchefa Biochemie, Netherlands
Magnesium chloride	Sigma-Aldrich, Germany
Magnesium chloride hexahydrate	Sigma- Aldrich, Germany
Magnesium sulphate heptahydrate	Merck, Germany
Methyl Alcohol	Lachner, Czech republic
Morpholinoethanesulphonic acid (MES)	Sigma-Aldrich, Germany
Murashige and Skoog basal salt mixture	Sigma-Aldrich, Germany
Myo-inositol	Sigma-Aldrich, Germany
N,N´-dimethylformamide	Sigma-Aldrich**,** Germany
Nicotinic acid	Duchefa Biochemie, Netherlands
Nitro blue tetrazolium chloride (NBT)	Sigma-Aldrich, Germany
Nonidet P-40	Sigma, Germany
Orange G	Sigma-Aldrich, Germany
Peptone from casein	Sigma-Aldrich, Germany
Ponceau S	Sigma-Aldrich, Germany
Pyridoxin	Duchefa Biochemie, Netherlands
SAVO	Bochemie, Czech republic
Silwet L-77	Lehle seeds, USA
Sodium dodecyl sulphate (SDS)	Duchefa Biochemie, Netherlands
Sucrose grade II	Sigma-Aldrich, Germany
Thiamine	Duchefa Biochemie, Netherlands
Trichloracetic acid (TCA)	Merck, Germany
Tris ultrapure	Duchefa Biochemie, Netherlands
Tween 20	Serva, Germany
Yeast extract	Sigma-Aldrich, Germany

3.1.6. Commercial kits for molecular biology

2D Quant Kit (GE Healthcare, USA): determination of protein concentration
GeneJet Plasmid Miniprep Kit (Fermentas, UK): isolation of plasmid DNA from bacteria
ImProm-II™ Reverse Transcription System (Promega, USA): RT-PCR
NucleoSpin Extract II (Machery-Nagel, Germany): extraction of DNA from gel
pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen, USA): TOPO® Cloning
pENTR™/D-TOPO® Cloning Kit (Invitrogen, USA): TOPO® Cloning
RNeasy Plant Mini Kit (Qiagen,Valencia, CA): RNA isolation

3.2. General laboratory equipment used

Centrifuge 5430 R	Eppendorf, Germany
Centrifuge MiniSpin plus	Eppendorf, Germany
Documentation system G:Box	Syngene, UK
E-centrifuge	Wealtec,USA
Elecroporator E.coli pulser nodel 1652102	Bio-Rad, USA
Incubator and Shaker	Edmund Büchler GmbH., Germany
Incubator INP 200	Memmert, Germany
Incubator	Stabilitherm™, USA
Master Cycler Gradient	Eppendorf, Germany
NanoDrop 1000 Spectrophotometer	Thermo scientific, USA
pH metr InoLab	WTW, Germany
Power pack 3000	Bio-Rad, USA
Refrigerated Incubator FTC 90I	Velp Scientifica, Italy
ShockPod™ Cuvette Chamber	Bio-Rad, USA
Silamat S5 Triturator	Vivadent, Liechtenstein
Spectrophotometer	Thermo electron, USA
Standard Power Pack P 25	Biometra, Germany
Thermomixer Comfort	Eppendorf, Germany
Thermoblock Cooling&Heating Block	Bioer, China
Ultracentrifuge Beckman L8-70M	Beckman, USA
Vortex Classic	Velp Scientifica, Italy
Water Bath GFL 1002	Scientific Instrument Centre, UK
Water Shaker GFL 1092	Scientific Instrument Centre, UK
Xp cycler	Bioer, China

3.3. Computational programmes and databases

- ***Sequence source:***

National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)
The CYP P450 Engineering Database (http://www.cyped.uni-stuttgart.de/)
P450 Database (http://drnelson.uthsc.edu/cytochromeP450.html)

- ***Sequence analysis and in vitro cloning:***

Vector NTI Suite 9.0.0 (Invitrogen, USA)
Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/)
Chromas Lite 2.01 (http://www.technelysium.com.au/chromas.html)

3.4. Methods and procedures

3.4.1. Outline of the approach used

To identify isoflavone synthase genes in the selected non-leguminous plants (Tab. 3.1.), a PCR-based strategy was used (Fig. 3.1.). After the collection of plant material, genomic DNA was extracted; PCR with degenerate and non-degenerate primers was carried out; amplified fragments were excised from the agarose gel; in the case of the degenerate primers, the fragments were cloned into the pGEM®-T Easy vector; finally, sequencing and sequence analysis were performed.

To conduct a pilot functional study with IFS (CYP93C18) from *Pisum sativum* L., *IFS* was first identified with the *CYP93C18*-specific primers; the gene was then cloned using Gateway™ Technology and used for the transformation of *Arabidopsis thaliana* (Col-0) by *Agrobacterium.* Subsequently, the *IFS* gene was over-expressed under the activity of CaMV35S promoter. The correct expression of *IFS* was verified at four levels (Fig. 3.2.). A similar procedure with a different destination vector was undertaken for the IFS intracellular localization *in vivo*.

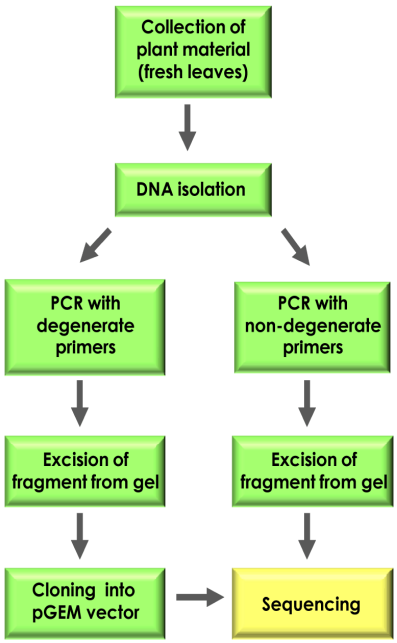


Fig. 3.1. Strategy for non-leguminous plants.

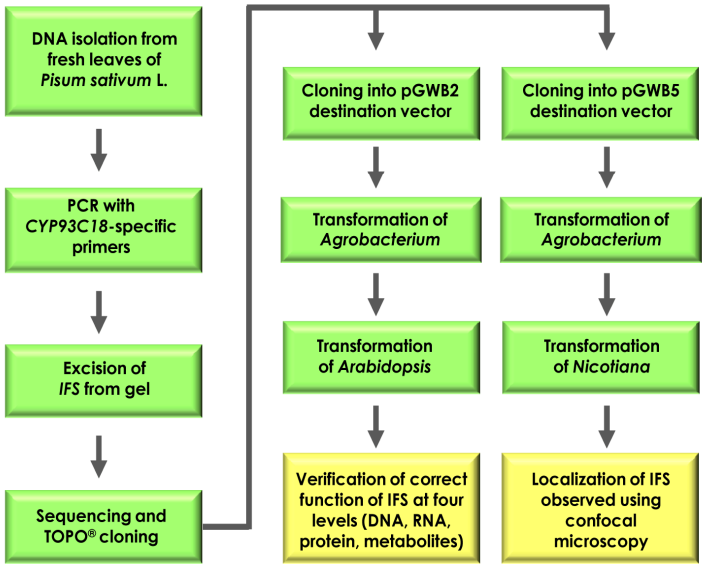


Fig. 3.2. Strategy for pilot study with *IFS* from *Pisum sativum* L. (CYP93C18).

3.4.2. Genomic DNA extraction

Genomic DNA from 100 mg of young leaves from the plants specified in 2.1.1. and from 100 mg of pulverized embryos of *Pachyrhizus tuberozus* (Lam.) Spreng. was extracted following the standard CTAB DNA extraction protocol (adapted from Weigel and Glazebrook, 2002). Fresh plant material was placed in 1.5 ml microcentrifuge tubes and frozen immediately in liquid nitrogen. The tissue was homogenised by intensive shaking with glass beads using Silamat S5 or by grinding in a frozen mortar with a pestle. It was then dissolved in 250 µl CTAB extraction buffer and incubated at room temperature for 20 min. Further, 250 µl of a chloroform:isoamyl alcohol mixture (24:1) was added, and the two phases – aqueous and organic – were subsequently separated by centrifugation for 10 min at 13 000 rpm. The upper aqueous phase was transferred into a new microcentrifuge tube containing 140 µl of isopropanol and spun down for 7 min at 13000 rpm. The supernatant was discarded. The pellet was washed with 1 ml of 70% ethanol and centrifuged for an additional 7 minutes. Ethanol was then removed by means of a water vacuum pump and its residues were evaporated in a thermostat at 55°C. The dried pellet was dissolved in 50 µl of double-distilled H_2O and incubated for 5 min at 55°C. The final concentration of extracted DNA was measured by means of a spectrophotometer NanoDrop, and samples were stored at -20°C.

- **CTAB extraction buffer:** 1.4M NaCl, 20 mM EDTA, 100 mM Tris (pH 8), 3% CTAB

3.4.3. Identification of isoflavone synthase gene (*IFS*)

3.4.3.1. Primers design

(1) *Pisum sativum* L.: *CYP93C18*-specific primers (Fig. 3.3.) were designed on the basis of the 1575-bp long *CYP93C18* sequence (Cooper *et al*., 2005) from the GenBank (NCBI). It was expected that the length of the amplified sequence would somewhat exceed 1575 bp, due to the presence of an intron.

Forward: 5´-CACCATGTTGGTTGAACTTGCACTTGCTT-3´
(CACC for TOPO® cloning)
Reverse 1: 5´-TTAAGAGGAAAGCAATTTAGCTGCT-3´
(used for stable expression)
Reverse 2: 5´-TGCAGAGGAAAGCAATTTAGCTGCT-3´
(stop codon TAA replaced by GCA coding alanine; used for transient expression)

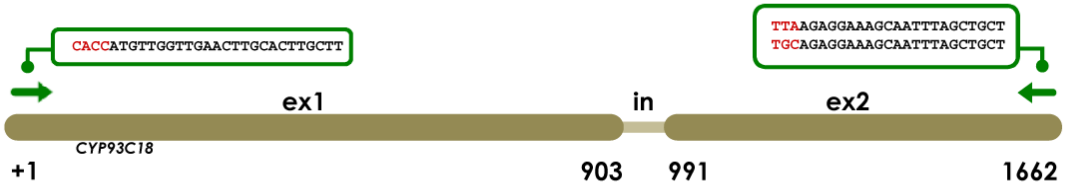


Fig. 3.3. *CYP93C18*-specific primers employed (in – intron, ex – exon).

(2) *Phaseolus vulgaris* L. and *Pachyrhizus tuberosus* (Lam.) Spreng.: Consensual primers were designed on the basis of multiple alignment of 30 known *IFS* sequences (primers constitute the first and last 26 nucleotides of *IFS*).

Forward: 5´-ATGTTGCTGGAACTTGCACTTGGTTT-3´
Reverse: 5´-TTAAGAAAGGAGTTTAGATGCAACGC-3´

(3) *All non-leguminous species*: Degenerate primers and their non-degenerate (consensual) versions were designed through a CODEHOP algorithm on the basis of highly conservative regions (Fig. 3.4.). The expected length of the fragments to be obtained was ca. 1000 bp.

Forward:
degenerate 5´-RAKWAKWGATGMAAGAAGTGTTGCCATTCC-3´
non-degenerate 5´-AATAAGAGATGCAAGAAGTGTTGCCATTCC-3´
Reverse:
degenerate 5´-YGAYCCTRTCRTTGAAAGGGTCATCAAGAA-3´
non-degenerate 5´-CGACCCTGTCGTTGAAAGGGTCATCAAGAA-3´

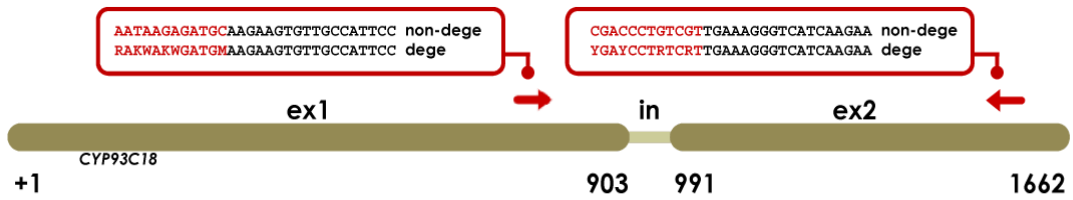


Fig. 3.4. Forward and reverse degenerate and non-degenetare primers employed (for clarity shown for the example of *CYP93C18*; in – intron, ex – exon).

The primers were synthetised by Sigma-Aldrich (Germany) or Genery Biotech (Czech Republic) and diluted in ddH_2O to achieve a concentration of 100 mM.

3.4.3.2. Polymerase chain reaction (PCR)

To identify *IFS* in the chosen plant species, PCR was carried out in each case with the respective primers and Taq DNA polymerase (Merci, Czech Republic). In the case of *CYP93C18*, Phusion high fidelity DNA polymerase (Finnzymes, Finland) was used for the gene identification and amplification, as it produces blunt-end PCR

products and its error rate is 50-fold lower than that of Taq polymerase. The optimal annealing temperatures of the primers were determined experimentally, using a wide gradient of temperatures.

The PCR mixtures consisted of:

TAQ DNA POLYMERASE		***PHUSION HIGH FIDELITY DNA POLYMERASE***	
Component	***Volume***	***Component***	***Volume***
10x Taq polymerase Buffer	2.5 µl	**5x Phusion HF Buffer**	5 µl
$MgCl_2$ (5 mM)	1 µl	**$MgCl_2$** (5 mM)	1 µl
dNTP mix (10 mM)	0.5 µl	**dNTP mix** (10 mM)	0.5 µl
Primer F (20 mM)	1 µl	**Primer F** (20 mM)	1 µl
Primer R (20 mM)	1 µl	**Primer R** (20 mM)	1 µl
DNA template (ca. 100 ng/µL)	1 µl	**DNA template** (ca. 100 ng/µL)	1 µl
Taq DNA polymerase	0.25 µl	**Phusion DNA polymerase**	0.3 µl
ddH_2O	17.75 µl	**ddH_2O**	15.2 µl
Final volume	25 µl	**Final volume**	25 µl

The reaction mixtures were distributed over the PCR plate, covered with aluminium sealing foil and placed into the PCR thermocycler.

The PCR conditions were as follows:

	TAQ DNA POLYMERASE	***PHUSION DNA POLYMERASE***	
Step	***Conditions***	***Conditions***	
Initial denaturation	94°C/7 min	98°C/30 s	
Denaturation	94°C/30 s	98°C/10s	35 cycles
Annealing*	gradient of 50-65°C/30s	58°C + 3°C/30s	
Extension	72°C/1 min	72°C/40s	
Final extension	72°C/10 min	72°C/10 min	

* For the purposes of verification of the presence of *CYP93C18* during the cloning process and in other confirmatory PCRs, the amplification of the gene was accomplished by Taq DNA polymerase at the determined optimal annealing temperature of 58.3°C and with time of extension 1.5 min.

3.4.3.3. Horizontal agarose gel electrophoresis

Agarose gel electrophoresis was used in order to separate and visualize PCR products and for the excision and isolation of gene fragments/genes of the desired length from the gel. 1% agarose gel was boiled in a microwave and once it was cooled to 55°C, ethidium bromide was added to a final concentration 0.1 µg/ml. After

solidification in a casting tray with a comb, the gel was submerged in 1x TAE buffer, and PCR mixtures with a loading buffer (12% v/v), and a molecular marker were loaded into the wells. The applied voltage was 80V or 110 V, depending on the size of the electrophoretic apparatus. After ca. 45 min, DNA bands were visualized by UV transilluminator and photographed using the documentation system G:Box (Syngene). All PCR products of interest were excised from the gel and purified by NucleoSpin Extract II kit, according to its manual.

- **50x TAE:** 24.2% (w/v) Tris, 10% (v/v), 0.5 M EDTA (pH 8), 5.7% (v/v) CH_3COOH
- **Agarose gel**: 1% (w/v) agarose diluted in 1x TAE buffer with 10 µg/ml ethidium bromide
- **Loading buffer for ELFO:** 0.7% (w/v) Orange G, 0.14 M EDTA (pH 8), 60% (v/v) glycerol
- **DNA molecular marker**: 1 µl GeneRuler™ 1 kb and 100 bp DNA Ladder (Fermentas, USA) mixed with 1 µl loading buffer and dissolved in 4 µl ddH_2O

3.4.3.4. DNA sequencing

All sequencing reactions were performed in the Laboratory of DNA Sequencing, Faculty of Science, Charles University in Prague, with the exception of *Phaseolus vulgaris* L. and *Pachyrhizus tuberozus* (Lam.) Spreng., of which the complete *IFS*s were sequenced in the Centre of DNA Sequencing, Institute of Microbiology AS CR.

3.4.4. Cloning of fragments obtained with degenerate primers

As the amplification using degenerate primers usually yields multiple PCR products, false priming is commonly observed when the obtained fragments are directly sequenced (Williams and Kane, 1993). Therefore, such fragments must be cloned into an appropriate vector and sequenced with suitable external vector-specific primers. PCR fragments obtained from examined non-leguminous species with degenerate primers, were cloned into pGEM®-T Easy Vector (Promega, USA; Fig. 3.5.).

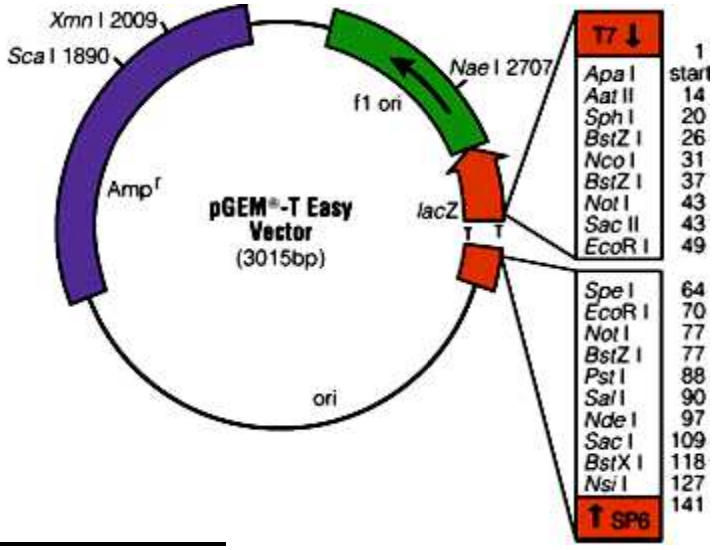


Fig. 3.5. pGEM®-T Easy Vector with the insertional site within the *lacZ* gene, enabling blue-white selection of transformants (adapted from Promega´s manual).

Reaction mixture

Component	Volume
PCR product	3.5 µl
2x Rapid Ligation Buffer	5 µl
pGEM-T Easy Vector (50 ng)	0.5 µl
T4 DNA Ligase	1 µl
Final volume	10 µl

The reaction mixture was incubated overnight at 4°C.

Transformation of competent cells

Chemical transformation of competent cells was performed. 25 µl of One Shot® TOP10 cells were added to 2 µl of the reaction mixture and incubated for 30 min on ice. The cells were then heat shocked for 45 s at 42°C and immediately plunged into ice. Two minutes later, 200 µl of S.O.C. medium were added and the tubes were shaken horizontally at 250 rpm for 1 hour at 37°C. The cells were subsequently spread over the selective plate with LB agar containing 100 µg/ml ampicilin and over whose surface 100 µL IPTG and 20 µL X-Gal (for blue-white selection) had been smeared. The plates were incubated overnight at 37°C. Typically, tens of white and blue colonies appeared. Several of the white colonies (presumably correctly transformed) were examined by colony PCR with the degenerate primers and pGEM-T Easy Vector-specific F (5´-TTTCACACAGGAAACAGCTATGA-3´) and R (5´- ACGGCCAGTGAATTGTA-ATACG-3´) primers. The PCR mixture and conditions were the same as above, with the following exceptions: the DNA template was represented by traces of the colony, and initial denaturation and denaturation steps were performed at 96°C. The successfully-transformed colonies were inoculated into 5 ml LB broth containing 100 µg/ml ampicilin and shaken (250 rpm) at 37°C overnight. The plasmid DNA was isolated by

the GeneJet Plasmid Miniprep Kit according to the manufacturer´s instructions, quantified, and sequenced with vector-specific primers.

- **IPTG stock solution (0.1M):** 1.2 g IPTG in 50 ml ddH_2O; stored at 4°C
- **X-Gal:** 100 mg in 2 ml N,N´-dimethylformamide; stored at -20°C, covered with aluminium foil

3.4.5. Cloning of putative *IFS* genes from *Phaseolus vulgaris* L. and *Pachyrhizus tuberosus* (Lam.) Spreng.

These two leguminous species were originally used as positive controls during the identification of *IFS* in the chosen non-leguminous species. However, the *IFS* sequences of both plants have not yet been identified. The appropriate PCR products were thus cloned in order to get the complete genes upon sequencing. As the outcome of cloning procedures tends to be somewhat unpredictable, the PCR product from *Pachyrhizus* was successfully cloned into pGEM-T Easy Vector, using the same protocol described in section 3.4.4., whereas the cloning of those from *Phaseolus* failed repeatedly. For this reason, pCR®8/GW/TOPO® (Fig. 3.6.) was chosen for TA cloning* of the PCR product from *Phaseolus*.

Reaction mixture

Component	*Volume*
PCR *product* excised from gel	1 µl
Salt solution provided within the kit	0.5 µl
pCR®8/GW/TOPO® provided within the kit	0.5 µl
ddH_2O	1 µl
Final volume	3 µl

The reaction mixture was incubated at room temperature for 10 min.

All the subsequent transformational steps were identical to those described in section 3.4.4., with the following exceptions: the selection antibiotic was spectinomycin (100 µg/ml); neither IPTG nor X-Gal were applied; the primers used for control PCR and sequencing were TOPO® vector-specific M13 F (5´-GTAAAACGACGGCCAGT-3´) and R (5´- AACAGCTATGACCATG-3´) primers.

*Our effort to obtain the gene by Phusion proofreading polymerase failed persistently, in contrast with those when Taq polymerase was used. In these circumstances we had to make do with the PCR products amplified by the latter. As this polymerase produces A-overhangs of the amplicons, TA cloning had to be carried out.

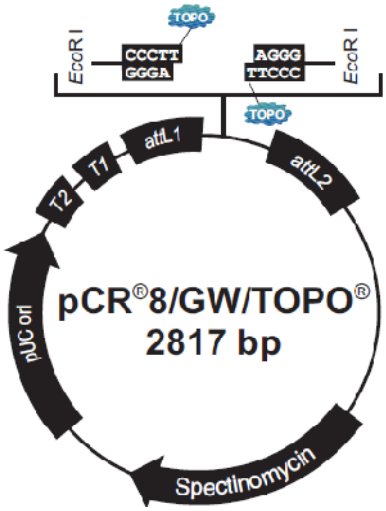


Fig. 3.6. pCR®8/GW/TOPO® Vector used for TA cloning of PCR product from *Phaseolus vulgaris* L. (adapted from Invitrogen´s manual).

3.4.6. Cloning and over-expression of *CYP93C18* using Gateway™ Technology

Gateway™ Technology (U.S. Patent 5,888,732, Life Technologies Inc., Invitrogen, USA) is a universal methodology for cloning PCR products and protein expression. It comprises three main successive steps:

(1) TOPO® cloning of the PCR product into an entry vector, to generate an **entry clone.**

(2) LR recombination between the entry clone and destination vector, to generate an **expression clone.**

(3) Introduction of the expession clone into the appropriate host and expression of the recombinant protein.

3.4.6.1. Directional TOPO® cloning of *CYP93C18*

Full-length and stop-codon-missing *CYP93C18* (both including an intron between positions 903 and 991) were cloned into the entry vector pENTR™/D-TOPO® (Invitrogen, USA). Both sequences carried four extra nucleotides CACC at the 5´end complemetary to GTGG overhang in the pENTR™/D-TOPO® (Fig. 3.7.) and had a blunt 3´end.

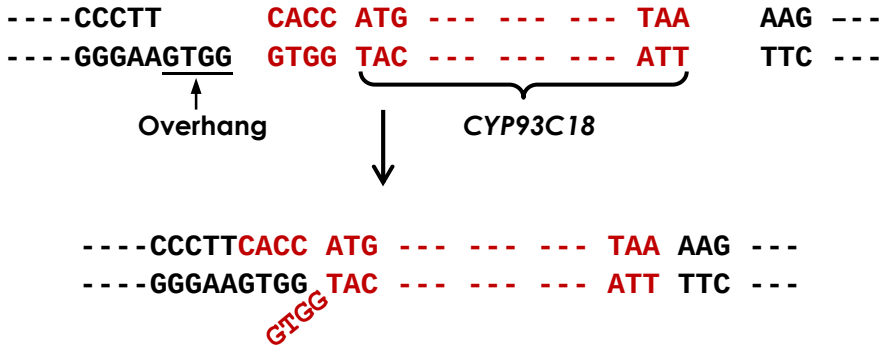


Fig. 3.7. TOPO® cloning principle (adapted from product manual).

The TOPO® cloning reaction was performed according to the kit manual, as follows:

Reaction mixture

Component	*Volume*
CYP93C18 excised from gel	1 µl
Salt solution provided within the kit	0.5 µl
pENTR™/D-TOPO® provided within the kit	0.5 µl
ddH_2O	1 µl
Final volume	3 µl

The reaction mixture was incubated at room temperature for 10 min.

Transformation of competent cells 1

Chemical transformation of competent cells was performed. 25 µl of One Shot® TOP10 were added to 3 µl of the reaction mixture and incubated for 30 min on ice. Then the cells were heat shocked for 45 s at 42°C and immediately tranferred to ice. 200 µl of the S.O.C. medium were added and the tubes were shaken horizontally at 250 rpm for 1 hour at 37°C. The cells were subsequently spread over the selective plate with LB agar and 50 µg/ml kanamycin, and incubated overnight at 37°C. Again, tens of white colonies appeared. Some of them were randomly chosen for colony PCR verification with *CYP93C18*-specific primers and Taq DNA polymerase (the PCR mixture and conditions were the same as indicated above, with these exceptions: DNA template – the trash of the colony, initial denaturation and denaturation steps – 96°C). The successfully transformed colonies were inoculated into 5 ml LB broth containing 50 µg/ml kanamycin, and shaken (250 rpm) at 37°C overnight. Entry clones, i.e. the TOPO® vector with the gene of interest inserted, were isolated from the competent cells by GeneJet Plasmid Miniprep Kit, according to the manufaturer´s instructions. For long-term storage, 700 µl of the bacterial culture were mixed with 300 µl of 50% (v/v) glycerol in a cryovial and deposited at -80°C. The acquired entry clones were analysed by PCR with *CYP93C18*-specific primers and TOPO® vector-specific primers M13F (5´-GTAAAACGACGGCCAGT-3´) and M13R (5´-AACAGCTATGACCATG-3´), under the same conditions as indicated in 3.4.3.2. above (Taq polymerase, annealing temperature of 58.3°C).

3.4.6.2. LR recombination reaction

To obtain an expression clone, LR recombination was performed, using Gateway® LR clonase™ II enzyme mix (Invitrogen, USA). In this step, *CYP93C18* (with and without stop codon), inserted into the TOPO® vector, was subcloned into the destination vector. LR Clonase recognized the recombination sites attL1 and attL2, which flank our gene of interest in the entry clone, cut the gene off and ligated it into the destination vector, thus replacing the gateway cassete attR1-ccdB-attR2. By way of destination binary vectors, pGWB2 and pGWB5 (obtained from Dr. Nakagawa, Shimane University, Japan) were employed for stable and transient expressions, respectively. These vectors contained the CaMV35S promoter upstream of the cloning site, kanamycin- and hygromycine-resistance genes and, in the case of pGWB5, the C-sGFP tag (Fig. 3.8.; Nakagawa *et al*., 2007).

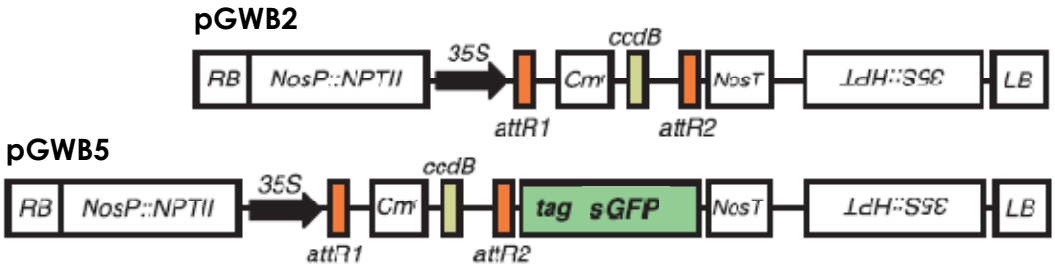


Fig. 3.8. Gateway binary vectors pGWB2 and pGWB5 (adapted from Nakagawa *et al*., 2007).

Before performing the LR reaction, the pGWB2 and pGWB5 vectors were linearised by digestion with XhoI (the XhoI site is unique in the vectors, situated as it is downstream of the attR1). The restriction reaction consisted of: 0.3 µl XhoI (Fermentas, USA), 1 µl 1x buffer R (Fermentas, USA), 2.5 µl pGWB2/5, 6.2 µl ddH_2O. The reaction was incubated for 2 hours at 37°C.

LR reaction mixture

Component	*Volume*
Entry clone (gene + / – stop codon)	1 µl
Destination vector (pGWB2/pGWB5)	2 µl
TE buffer	1 µl
Gateway® LR clonase™ II enzyme mix	1 µl
Final volume	5 µl

- **TE buffer:** 10 mM Tris, 1 mM EDTA; pH 7.5

The reaction mixture was incubated at room temperature overnight. To terminate the reaction, 2 µl of proteinase K (Invitrogen, USA) were added and samples incubated for 10 min at 37°C.

Transformation of competent cells 2

Chemical transformation of competent cells was performed. 25 µl of *Alpha*-Select Gold Efficiency were added to 5 µl of the reaction mixture. The procedure (including long-term storage) continued as already described under *Transformation 1* above, except for the fact that LB plates and subsequently LB broth contained 50 µg/ml kanamycin and 50 µg/ml hygromycin, as selection antibiotics. Both of the acquired expression clones were analysed by PCR with *CYP93C18*-specific primers and pGWB vectors-specific primers pGWBF and (5´-TCATTTCATTTGGAGAGAACACG -3´) and pGWBR (5´-CCACTTTGTACAAGAAAGCTGG-3´), under the same conditions as indicated in 3.4.3.2. above (Taq polymerase, annealing temperature of 58.3°C).

3.4.6.3. Transformation of *Agrobacterium tumaefaciens*

The expression clones obtained were introduced into *Agrobacterium tumaefaciens* strain GV3101 by electroporation. 1 µl of pGWB2::IFS and pGWB5::IFS was mixed with 50 µl of *Agrobacterium* in a sterile electroporation cuvette. This was then placed in an electroporator and a voltage of 2.5 kV was applied. 800 µl of LB broth were added immediately, and in each case the solution was transferred to a 1.5 ml microcentrifuge tube and incubated for 2 hours at 28°C. 200 µl cultures were spread on YEB-plates containing 50 µg/ml kanamycin, 50 µg/ml hygromycin, 50 µg/ml gentamycin and 100 µg/ml rifampicin and incubated overnight at 28°C, covered in aluminium foil. The following day, colony PCR was performed (under conditions specified in 3.4.3.2. above). For long-term storage, 700 µl of the *Agrobacterium* culture in YEB medium containing the above-mentioned antibiotics, were mixed with 300 µl of 50% (v/v) glycerol in a cryovial, and deposited at -80°C.

3.4.7. Stable expression of CYP93C18

3.4.7.1. Transformation of *Arabidopsis thaliana*

To obtain stable transformants of *Arabidopsis* over-expressing *CYP93C18*, pGWB2::IFS expression clones were introduced via transformed *Agrobacterium* into *Arabidopsis* flower buds, using the floral dip method (Clough and Bent, 1998). A

colony of *Agrobacterium* with a confirmed presence of *CYP93C18* was inoculated into 150 µl of YEB medium containing 50 µg/ml kanamycin, 50 µg /ml hygromycin, 50 µg/ml gentamycin and 100 µg/ml rifampicin, and shaken overnight at 28°C. The culture was then centrifuged in Beckman centrifuge tubes (250 ml) for 20 min at 4,500 rpm at 4°C in the Beckman centrifuge. Pelleted cells were resuspended in a mixture of sucrose (10% w/v) and Silwet L-77 (0.05% v/v) to reach OD_{600} from 1.0 to 1.5 (measured by spectrophotometer Thermo electron). Flower buds of eight *Arabidopsis* plants were dipped for several seconds in 200 µl of the resulting solution, with gentle agitation. The transformed plants were covered in plastic bags and placed in the dark overnight. The following day, the plants were transferred into a growth chamber. The procedure was repeated with the same plants five days later.

3.4.7.2. Selection of transformants

2-3 weeks after transformation, the seeds of the transformed plants were harvested and surface-sterilized. The sterilization procedure comprised the following steps: (1) placing the seeds in the freezer overnight, (2) shaking the seeds in 70% ethanol for 5 min, (3) discarding the supernantant and shaking the seeds in 10% (v/v) SAVO with 0.1% (v/v) nonidet for 20 min, and (4) discarding the supernatant and washing the seeds 5 times in ddH_2O. About 2000 such sterilized seeds were selected on MS-plates containing 50 µg/ml hygromycin and cultivated at 21°C with a 16-hour photoperiod. The hygromycin-resistant four-leaf seedlings were transferred into Jiffy peat pots (Jiffy Products (N.B.) Ltd., New Brunswick), allowed to grow at 21°C with a 16-hour photoperiod and later analysed. The same selection procedure was repeated over 2 generations to obtain homozygous transformants.

3.4.7.3. Analysis of transgenic *Arabidopsis thaliana*

At all the analytical levels described below, the same procedures were also carried out with *Pisum sativum* L. and with wild-type *Arabidopsis*, as positive and negative controls, respectively.

3.4.7.3.1. Level 1: DNA

DNA was extracted from 100 mg of leaves of 10 T1 and 20 T2 randomly-selected transformants, as described in 3.4.2 above, and control PCR, with *CYP93C18*-specific primers and Taq polymerase under standard conditions, was carried out.

3.4.7.3.2. Level 2: RNA

Total RNA was extracted from 100 mg of the leaves of individual plants in which the presence of *IFS* gene was confirmed, using RNeasy Plant Mini Kit. The RNA concentration was measured by NanoDrop. RT-PCR was then performed, using the ImProm-II™ Reverse Transcription System (Promega, USA), according to the manufacturer´s instructions. The denaturation reaction mixture consisted of RNA (up to 1 µg/reaction), 1 µl oligo $(dT)_{15}$ and nuclease-free water, to achieve a final volume of 5 µl. This mixture was incubated at 70 °C for 5 min, immediately chilled on ice (for 10 min) and added to the RT-PCR reaction.

RT-PCR reaction mixture

Component	*Volume*
5x ImProm-II™ 5x reaction buffer	4 µl
$MgCl_2$	4 µl
dNTP mix	1 µl
Recombinant Rnasin® Ribonuclese inhibitor	1 µl
ImProm-II™ Reverse Transcriptase	1 µl
Nuclease-Free H_2O	4 µl
Final volume	15 µl

RT-PCR reaction

Step	*Conditions*
Annealing	25°C/5 min
Extension	42°C/1 hour
Inactivation	70°C/15 min

The final PCR amplification was carried out with *CYP93C18*-specific primers and Taq polymerase under standard conditions (as above).

3.4.7.3.3. Level 3: Proteins

Protein Extraction. Total protein was first extracted in accordance with the procedure due to Wang *et al.* (2006), from 100 mg of leaves from confirmed IFS-transgenic *Arabidopsis.* In brief, (1) plant material was ground in a mortar with a pestle, (2) washed with 10% TCA/acetone, (3) washed with 80% methanol with 0.1 M ammonium acetate, (4) washed with 80% acetone, and (5) air-dried overnight; (6) proteins were extracted using 1:1 phenol (pH 8)/SDS buffer and precipitated in 80% methanol with

0.1 M ammonium acetate, (7) the pellet was washed in 100 % methanol and (8) in 80% acetone, and (9) air-dried overnight. The extracted proteins were quantifed using the 2-D Quant kit, according to the manufacturer´s protocol.

- **SDS buffer**: 30% (w/v) sucrose, 2% (w/v) SDS, 1.21 % (w/v) TRIS, 5% (v/v) mercaptoethanol

Protein Separation. The extracted proteins were separated using 1-D SDS-PAGE. Samples were dissolved in the STM sample buffer, stained with bromphenol blue and denaturated by boiling for 5 min. SDS-PAGE was then performed in 10% resolving and 4% stacking gels, using the Multigel-Long apparatus (Biometra, Germany). A voltage of 80 V was applied for 1 hour (Bio-Rad Power pack 3000), and 180 V were then applied for a further 4 hours or so at 12.5°C.

- **STM sample buffer**: 2% (w/v) SDS, 6% (w/v) TRIS (pH 6.8), 0.1% (v/v) glycerol, 5% (v/v) mercaptoethanol
- **SDS-PAGE Molecular Weight Standards**, Broad Range (Bio-Rad, USA).

Western blotting and immunodetection. The separated proteins were tranferred onto a nitrocellulose membrane (Serva, Germany) using the Bio-Rad Mini Trans-Blot® Cell. The transfer was conducted at 4°C, initially overnight under a voltage of 30 V, and at 60 V for an ensuing hour. The nitrocellulose membranes were then reversibly stained with Ponceau S, to control the transfer efficiency. For subsequent immunodetection, a peptide sequence derived from a highly-conservative region of IFS was used to prepare an IFS-specific antibody. Its epitop surface localization was confirmed by means of protein modelling in Modeller9v5 (Eswar *et al*., 2006). The peptide NH_2-DPKYWKRPLEFRPER (according to Yu *et al.,* 2000), conjugated with KLH (Davids biotechnologie, Regensburg), was then used for the immunization of a rabbit (Department of Biological Control, Institute of Physiology AS CR, Prague), according to their immunization schedule. The immunodetection was performed with total proteins on the nitrocellulose membranes and with IFS antibodies, 30 days after immunization. The primary antibody was diluted 1:200 in a TBST buffer with 5% non-fat dry milk. The secondary Anti-rabbit Goat IgG, conjugated to alkaline phosphatase (Sigma-Aldrich, Germany), was used in 1:15,000 dilution in a TBST buffer with 1% BSA. Detection of IFS was carried out by submerging the nitrocellulose membrane

(Serva, Germany) in an AP buffer with 0.37 mM BCIP and 0.37 mM NBT, and blue bands were observed after the membrane dried.

- **Ponceau S**: 2% (w/v) Ponceau S dissolved in 3% (v/v) TCA
- **TBST buffer**: 0.242% (w/v) TRIS, 0.8% (w/v) NaCl, 0.05% (w/v) Tween 20
- **AP buffer**: 1.211% (w/v) TRIS, 0.584 (w/v) NaCl, 0.101 %(w/v) $MgCl_2$ .6 H_2O

3.4.7.3.4. Level 4: Metabolites

Measurements were carried out by Ing. Petra Mikšátková at the Department of Chemistry of Natural Compounds at the Institute of Chemical Technology in Prague.

Lyophilised shoots and dried seeds were ground. 0.4 g of the plant material, together with 8 ml of 80% ethanol, were left for 14 days at room temperature, with occasional agitation. The extracts were then centrifuged and filtered through a 17 mm, 0.45 µm PTFE syringe filter. 2.5 ml of extracts were dried and then dissolved in 0.3 ml of 40% methanol, for HPLC-ESI-MS analysis. The HPLC-MS tandem consisted of a Hewlett Packard (HP/Agilent Technologies, USA) 1100 HPLC series and a HP Mass selective detector (G1946A), and was controlled by means of ChemStation software (revision A 07.01). A LiChroCART® 125-4 mm HPLC-Cartridge with Prospher® STAR RP-18 endcaped (5 µm), was used as a column (Merck, Germany). Mobile phases were 40% methanol (solvent A) and 100% methanol (solvent B), both with 0.5% acetic acid. The following gradient was employed (all steps linear): 0 min, 100:0 (A:B); 5 min, 80:20; 15 min, 55:45; 20 min 0:100; 23 min, 0:100; 27 min, 100:00 and at 27 min stop followed by 3 min post-run. The flow-rate was 0.8 ml/min, the temperature of the column thermostat was set at 25°C and the injection volume was 20 µl. The mass spectrometer was operating in the positive ESI mode. Individual isoflavones were identified by comparing their retention times (t_R) and molecular ions $[M+H]^+$ with those of standards.

3.4.8. Transient expression of CYP93C18

3.4.8.1. Transformation of *Nicotiana benthamiana*

In order to visualize IFS subcellular localization, pGWB5::CYP93C18::GFP was infused into the *Nicotiana benthamiana* leaves through infiltration by syringe injection with the inoculum of transformed *Agrobacterium* (Li *et al*., 2009). Briefly, the transformed *Agrobacterium* was inoculated into 5 ml YEB medium with appropriate antibiotics (see 3.4.6.3.) and shaken at 28 °C overnight. The cultures were then spun

down at 8000 rpm for 5 min. Pelleted cells were resuspended with 1 ml infiltration medium and briefly centrifuged. The pellet was resuspended in the infiltration medium to OD_{600} 0.1 and incubated for 1.5 hours in the dark at room temperature. The lower epidermis of young leaves of *Nicotiana benthamiana* was gently grazed by a sterile needle, and *Agrobacterium* solution (500 µl per one leave) was injected by means of a syringe into the leaves. Transformed plants were cultivated in the dark at room temperature overnight and then moved to a growth chamber. As a positive control, *Agrobacterium* strain C58C1 carrying the pBIN m-gfp5-ER plant binary vector (transformed culture obtained from Dr. Jan Petrášek, IEB ASCR), was used. It encodes for the ER-localized GFP variant mGFP5-ER, a thermotolerant derivative of mGFP4-ER (Haseloff *et al*., 1997), and contains a C-terminal ER retention signal sequence (HDEL). As a negative control, *Agrobacterium* strain GV3101 carrying pGWG5 without any insert (expression of free sGFP), was employed. Both of the control proteins were transiently expressed in *Nicotiana* leaves, using the same protocol.

• **Infiltration medium**: 10 mM MES, 10 mM $MgCl_2$, 200 µM acetosyringon (200 mM stock solution in DMSO)

3.4.8.2. Analysis of transgenic *Nicotiana bethamiana*

Nicotiana plants were analysed 2-3 days after the transformation. The *Agrobacterium* injection spots on the *Nicotiana* leaves were excised and subjected to immediate observation by means of confocal laser scanning microscopy (LSM Zeiss 5 Duo, Germany), using the confocal microscope with the 488 nm laser line of the ArKr laser.

3.4.9. Modelling of CYP93C18

The CYP93C18 protein structure was modelled in collaboration with Ing. Roman Pleskot from the Institute of Experimental Biology AS CR, Prague.

The appropriate templates for homology modelling of the CYP93C18 protein structure were found using the Psipred threading algorithm (Bryson *et al.*, 2005). The three-dimensional model was then generated on the basis of the resulting alignment, by means of Modeller9v5 (Eswar *et al*., 2006). The Adaptive Poisson-Boltzmann Solver (APBS) was used for the evaluation of the electrostatic properties of the modelled structure (Baker *et al*., 2001). Molecular graphics images were produced by the UCSF Chimera package, the University of California, San Francisco (Pettersen *et al*., 2004).

4. RESULTS

4.1. Identification of isoflavone synthase genes (*IFS*)

On the basis of the extensive literature dealing with the detection of isoflavonoids in various plant families, we selected 33 non-leguminous plant species from 14 different families, from which 8 families were isoflavonoid producers and 6 were not (Tab. 4.1.; non-producing families marked with an asterisk; according to Lapčík, 2007). Two control leguminous plants whose IFS sequences are not in the GenBank as yet – *Phaseolus vulgaris* L. and *Pachyrhizus tuberozus* (Lam.) Spreng. – were also examined, with a high probability of the presence of *IFS*.

4.1.1. Identification of *IFS* in the selected non-leguminous species

To identify the *IFS* orthologues, PCR was performed with degenerate primers, designed within the most conservative regions discovered by multiple alignment of known *IFS*s (section 3.4.3.1.) and with the genomic DNA extracted from chosen plants.

In nine of the 33 plant species examined, no PCR product was obtained, even though numerous optimizations attempts were made (including repeated DNA extraction, various concentrations of PCR components, gradient PCR etc.). In the remaining species, PCR products of different lengths appeared and were further investigated (Tab. 4.1.).

However, the subsequent procedures leading to sequencing met with several complications, ranging from failure during the cloning of the fragments into the pGEM®- T Easy Vector, to erroneous sequencing with no sequence being obtained. In those instances where cloning failed persistently (nine species out of the remaining 24), the fragment was sequenced with the non-degenerate version of the degenerate primers originally used, once control PCR with those non-degenerate primers had been carried out.

Regrettably, a BLAST search (blastn suite 2.2.23+; Altschul *et al.*, 1997) undertaken after the obtained fragments were sequenced, revealed that none of the PCR products from non-leguminous species were homologous with *IFS* genes from the GenBank. What is even more peculiar is the fact that more than half of the acquired

sequences displayed no significant homology to any sequence deposited in the GenBank. In contrast, both sequences amplified from *Pachyrhizus* (900 bp) and *Phaseolus* (750 bp) displayed a significant homology to *Glycine soja – IFS 1* (gb|EU391492|; similarity 89%) and *Pueraria montana IFS* (gb|EU737110|; similarity 88%) respectively (Fig. 4.1. and Tab. 4.1.). These results suggest the utility of the designed primers for the identification of *IFS* orthologues in more species from the Fabaceae family, but the primers did not work well in phylogeneticaly distant taxa.

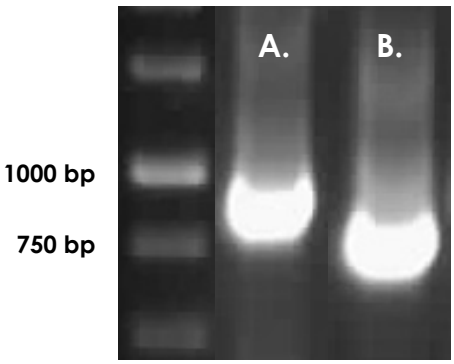


Fig. 4.1. Electrophoretogram of the fragments from *Pachyrhizus tuberosus* (A) and *Phaseolus vulgaris* (B), obtained after PCR with degenerate primers. These fragments were later confirmed to be homologous to known *IFS* genes.

An interesting result was achieved when *CYP93C18*-specific primers (see section 4.2.) were used with genomic DNA isolated from *Humulus lupulus* and *Iris sp*. The lengths of the PCR products, 1700 bp and 1500 bp, respectively, were similar to those of the product from the control plant *Pisum sativum* (Fig. 4.2.). However, upon sequencing, these promising results turned out to be misleading: the sequences were not homologous to any known *IFS* genes (Tab. 4.1.).

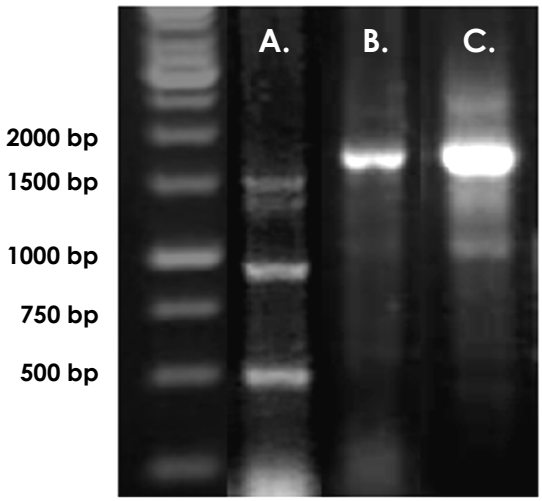


Fig. 4.2. Electrophoretogram of the fragments from *Iris* sp. (A), *Humulus lupulus* (B) and control plant *Pisum sativum* (C), obtained after PCR with *CYP93C18*-specific primers. The products (A) and (B) were shown not to be homologous to hitherto known *IFS* genes. In the case of *Pisum*, the sequence obtained was 99% homologous to *IFS* (CYP93C18) from GenBank.

Degenerate primers

Plant family	Plant species	PCR product length	pGEM cloning	Sequencing/primers	Blast search (% max. identities)
Annonaceae*	***Annona cherimolia***	1000 bp	Yes	Yes/pGEM	No similarity found
	Annona muricata	800 bp	Yes	Yes/pGEM	Liriodendron tulipifera chloroplast genome; 85%
Cannabaceae	***Cannabis sativa***	X	X	X	X
	Humulus lupulus	1000 bp	No	Yes/non-dege	No similarity found
		700 bp	No	Yes/non-dege	Vitis vinifera contig VV79X002481.3; 69%
Chenopodiaceae	***Beta vulgaris***	1000 bp	Yes	Yes/pGEM	No sequence obtained
Ephedraceae*	***Ephedra sinica***	750 bp	No	Yes/non-dege	Nasturtium officinale chloroplast DNA; 76%
Erythroxylaceae	***Erythroxylon coca***	1500 bp	Yes	Yes/pGEM	No similarity found
		1200 bp	Yes	Yes/pGEM	Populus trichocarpa predicted protein; 75%
Iridaceae	***Iris sp.***	X	X	X	X
Lamiaceae*	***Hyssopus officinalis***	750 bp	No	Yes/non-dege	Petunia integrifolia S-2 RNase and SLF2; 75%
	Lamium album	1000 bp	Yes	Yes/pGEM	No similarity found
	Levandula angustifolia	1100 bp	Yes	Yes/pGEM	No similarity found
		800 bp	Yes	Yes/pGEM	No similarity found
	Mentha piperita	X	X	X	X
	Ocimum basilicum	900 bp	Yes	Yes/pGEM	Solanum lycopersicum (chromosome 8); 72%
	Origanum vulgare	600 bp	Yes	Yes/pGEM	No similarity found
	Pogostemon cablin	1800 bp	Yes	Yes/pGEM	No similarity found
		800 bp	Yes	Yes/pGEM	No similarity found
		550 bp	Yes	Yes/pGEM	Arabidopsis lyrata clone SINE9 transposon; 96%
	Rosmarinus officinalis	1500 bp	Yes	Yes/pGEM	No similarity found
	Salvia officinalis	1000 bp	No	Yes/non-dege	Lotus japonicus (chromosome 4); 68%
	Scutellaria baicaliensis	750 bp	No	Yes/non-dege	No sequence obtained
	Thymus serpyllum	X	X	X	X
	Thymus vulgaris	X	X	X	X

Tab. 4.1. Table of selected non-leguminous plants and control leguminous plants examined. Plant families not producing isoflavonoids are marked with an asterisk (*).
The table continues on the next page.

Degenerate primers - continued

Plant family	Plant species	PCR product length	pGEM cloning	Sequencing/primers	Blast search (% max. identities)
Lauraceae*	***Persea americana***	800 bp	Yes	Yes/pGEM	No similarity found
Pedaliaceae*	***Sesamum indicum***	1100 bp	Yes	Yes/pGEM	Nicotiana tabacum mitochondrial DNA; 87%
Ranunculaceae*	***Heleborus niger***	X	X	X	X
Rubiaceae	***Coffea arabica***	X	X	X	X
Rutaceae	***Ruta graveolens***	1500 bp	No	Yes/non-dege	Helianthus petiolaris retrotransposon Ty3; 80%
Solanaceae	***Capsicum annuum***	1000 bp	Yes	Yes/pGEM	Solanum tuberosum (chromosome 11); 73%
	Capsicum frutescens	1200 bp	Yes	Yes/pGEM	No similarity found
	Nicotiana rustica	1500 bp	Yes	Yes/pGEM	Petunia integrifolia S-2 RNase and SLF2, 74%
		700 bp	Yes	Yes/pGEM	Solanum lycopersicum (chromosome 2); 68%
	Physalis peruviana	1000 bp	No	Yes/non-dege	No similarity found
	Solanum lycopersicum	600 bp	No	Yes/non-dege	Solanum lycopersicum (chromosome 8); 81%
	Solanum muricatum	X	X	X	X
	Solanum melongena	800 bp	Yes	Yes/pGEM	No similarity found
Zygophyllaceae	***Tribulus terestris***	X	X	X	X
Fabaceae (control)	***Pachyrhizus tuberosus***	900 bp	No	Yes/non-dege	Glycine soja – Isoflavone synthase 1; 89%
	Phaseolus vulgaris	750 bp	No	Yes/non-dege	Pueraria montana – Isoflavone synthase; 88%

***CYP93C18*-specific primers**

Plant family	Plant species	PCR product length	pGEM cloning	Sequencing/primers	Blast search (% max. identities)
Cannabaceae	***Humulus lupulus***	1700 bp	Yes	Yes/pGEM	No similarity found
Iridaceae	***Iris sp.***	1500 bp	Yes	Yes/pGEM	Arabidopsis lyrata SINE8 transpozone; 94%
		1400 bp	Yes	Yes/pGEM	No similarity found
		900 bp	Yes	Yes/pGEM	Humulus lupulus microsatellite; 69%
		500 bp	Yes	Yes/pGEM	Arabidopsis lyrata Gypsy21 transposon; 99%
Fabaceae (control)	***Pisum sativum***	1700 bp	Yes	Yes/pGEM	Isoflavone synthase (CYP93C18); 99%

4.1.2. Identification of complete *IFS* sequences from *Pachyrhizus* and *Phaseolus*

Having obtained the partial *IFS* sequences from *Pachyrhizus tuberosus* and *Phaseolus vulgaris,* as detailed in section 4.1.1. above, our effort continued towards the objective of identifying their complete *IFS* genes, which are hitherto unknown.

Both above-mentioned leguminous species are well-known producers of isoflavonoids. As reported, five isoflavonoid phytoalexins – phaseollin, phaseollidin, phaseollinisoflavan, coumestrol and kievitone (Gnanamanickam, 1979) – and a further 8 isoflavonoids with phytoestrogenic activity – daidzein, glycitein, genistein, formononetin, biochanin A together with glycosides daidzin, glycitin and genistin (Nakamura, 2000) – have been detected in *Phaseolus vulgaris* L. In the genera *Pachyrhizus*, relatively high levels of the insecticidal isoflavonoids rotenone and pachyrhizine have been found (Lackhan, 1994).

Using genomic DNA as a template, PCR was performed with primers designed on the basis of the consensual sequence derived from the alignment of known *IFSs* (the first and the last 26 nucleotides). The PCR products of the appropriate length (ca. 1800 bp) were obtained in both cases (Fig. 4.3.). The putative *IFS* genes from *Phaseolus* and *Pachyrhizus* were cloned into pCR8®/GW/TOPO® Vector and pGEM®-T Easy Vector, respectively. The cloned plasmids were isolated, PCR-verified (Fig. 4.4.), and sequenced from both 5´- and 3´-ends with the appropriate vector-specific primers (M13 and pGEM, respectively).

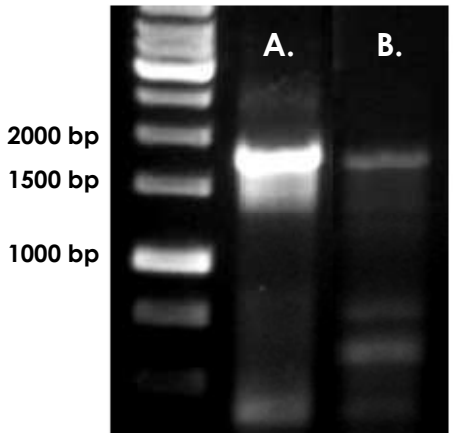


Fig. 4.3. Electrophoretogram of PCR products obtained from *Phaseolus vulgaris* (A) *Pachyrhizus tuberosus* (B), of the desired length of ca.1800 bp.

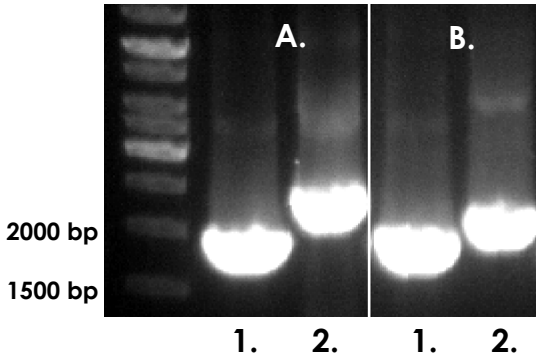


Fig. 4.4. Electrophoretogram confirming the presence of the right inserts in isolated plasmids. (A) *Phaseolus vulgaris*, (B) *Pachyrhizus tuberosus*. (1) PCR products (~1800 bp) obtained with internal consensual primers, (2) PCR products (>1800 bp) obtained with external vector-specific primers.

4.1.2.1. Characterization of IFS from *Phaseolus vulgaris* L.

The CDS of 1566 bp in length (without intron) amplified from *Phaseolus* was obtained upon sequencing and subsequent editing using Vector NTI 9.0.0. BLAST examination of the generated nucleotide sequence showed the highest homology with the isoflavone synthase 1 mRNA from *Vigna unguiculata* (gb|EU616497|; Kaur and Murphy, 2008, unpublished) with the E-value of 0.0 and identities 1481/1566 (94.6%). The genomic sequence contained a 294-bp intron, with the splice junction between nucleotide positions 903 and 904 of the coding region. *In silico* translation resulted in a protein sequence of 521 amino-acids. Accordingly, this sequence displayed the highest homology to the *Vigna unguiculata* IFS protein deduced from the above-mentioned GenBank accession (Fig. 4.5.). The alignment parameters were as follows: E-value = 0.0; Identities = 489/521 (93.9%); Similarity = 507/521 (97.3%). It must be noted that the first and last four amino-acids of the sequence are derived from the primers used for the original amplification.

```
Pvul MLLELALGLLVLALFLHLRPTPTAKSKALRHLPNPPSPKPRLPFIGHLHLLKDKLLHYAL  60
Vuni MLLEITIGLLVLALFLHLRPTPTAKSKALRHLPNPPSPKPRLPFIGHLHLLKDKLLHYAL  60

Pvul IDLSKRHGPLFSLYFGSMPTVVASSPELFKLFLQTHEAASFNTRFQTSAIRRLTYDNSVA  120
Vuni IDLSKTYGPLYSLYFGSMPTVVASSPELFKLFLQTHEAASFNTRFQTSAIRRLTYDNSVA  120

Pvul MVPFGPYRKFIRKLIMNDLLNATTVNKLRPLRTQEIRKFLKVMAQSAKAQQPLNVTEELL  180
Vuni MVPFGPYWKFIRKLIMNDLLNATTVNKLRPLRTQQIRKFLKVMAQSAQAQQPLNVTEELL  180

Pvul KWTNSTISMMMLGEAEEIRDIARDVLTIFGEYSLTNFIWPLNKLKLGKYENRTEEIFNKF  240
Vuni KWTNSTISMMMLGEAEEIRDIAREVLKIFGEYSLTDFIWPLKKLKFGQYEKRIDEIFNKF  240

Pvul DPIIEKVIKKRREIVRRRKNGEVVAEEQSGVFLDTLLEFAEDETMEIKITDEQIKGLVVD  300
Vuni DPVIERVIKKRREIMRRRKNGEAVEEEQSGVFLDTLLQFAEDETMEIKITKEQIKGLVVD  300

Pvul FFSAGTDSTAVATEWALAELINNPRVLQKAREEVYSVVGKDRLVDEVDTQNLPYIRAIVK  360
Vuni FFSAGTDSTAVATEWALAELINNPRVLQKAREEVYSVVGKDRLVDEVDTQNLPYIRAIVK  360

Pvul ETFRMHPPLPVVKRKCVEECEIDGYVIPEGALILFNVWAVGRDPKYWNRPLEFRPERFLE  420
Vuni ETFRMHPPLPVVKRKCVEECEIEGYVIPEGALILFNVWAVGRDPKYWDRPLEFRPERFLE  420

Pvul SEAEGEAGPLDLRGQHFTLLPFGSGRRMCPGVNLATSGMATLLASVIQCFDLQVVGSQGQ  480
Vuni TGAEGEAGPLDLRGQHFTLLPFGSGRRMCPGVNLATSGMATLLASVIQCFDLQVVGPQGQ  480

Pvul ILKGNDAKVSMEERGGLTVPRAHNLECVPVARTSVASKLLS* 521
Vuni ILKGNDAKVSMEERAGLTVPRAHNLECVPVARTSVAAKLLS* 521
```

Fig. 4.5. ClustalW alignment of deduced amino-acid sequences of IFS from *Phaseolus* (Pvul) and *Vigna* (Vuni). The mismatched amino-acids which do not have similar properties are coloured in red; the mismatched amino-acids with similar properties are highlighted in blue.

4.1.2.2. Characterization of IFS from *Pachyrhizus tuberozus* (Lam.) Speng.

As in the previous case, the CDS of length 1560 bp (without intron) from *Pachyrhizus* was established upon sequencing and subsequent editing using Vector NTI 9.11. BLAST search of the obtained nucleotide sequence displayed the highest homology to the isoflavone synthase mRNA *Pueraria montana* var. lobata (gb| AF462633|; Jeon and Kim, 2001, unpublished), with the E-value of 0.0 and identities 1405/1566 (89.7%). The sequence contained a 243-bp intron, with the splice junction between nucleotide positions 897 and 898 of the coding region. The deduced protein sequence of 519 amino-acids showed the highest homology with the isoflavone synthase 2 from *Vigna unguiculata* (gb|ACC77197|; Kaur and Murphy, 2008, unpublished) (Fig. 4.6.). The alignment parameters were as follows: E-value = 0.0; Identity = 470/522 (90.0%); Similarity = 495/522 (94.8%); Gaps = 3. It must again be noted that the first and last four amino-acids of the sequence gained are derived from the primers used for the original amplification.

```
Ptub MLLELALGLFVLALFLHLRPRPSAKSKALRHLPNPPSPKPRLPFIGHLHLLDKTLLHHSL 60
Vuni MLLEIAIGLLVLGLFLHLRPTPSAKSKALRHLPNPPSPKPRLPLIGHLHLLKDQLLHHSL 60

Ptub ISLSQRYGPLYSLYFGSMPTVVASTPELFKLFLQTHEATSFNTRFQTSAIKRLTYNNSVA 120
Vuni IDLSKRYGPLYSLYFGSMPTVVASTPELFKLFLQTHEAASFNTRFQTSAIKRLTYDNSVA 120

Ptub MVPFGPYWKFIRKLIMNDLLNATTVNKLRPLRTQEIRKVLRVLAQSAEAQQSLNVTEELL 180
Vuni MVPFGPYWKFIRKLIMNDLLNATTVNKLRPLRSHEIRKVLRVLAQSAEAQQPLNVTEELL 180

Ptub KWTNNTISMMMLGEAEEVRDMAREAVKIFGEYSLTDFIWPLKKLKFGQYEKRIEEIFNKF 240
Vuni KWTNNTISMLMLGEAEEVRDLARETVKIFGEYSLTDFIWPLKKLKFGKYEKRIDEIFNKF 240

Ptub DPAIEKVIKKRREIVRRRKNGEVVEGEASGVFLDTLLEFAEDETVEIKITKEQIKGLVVD 300
Vuni DPVIEKVIKKRQEIVRRRKNGEVVEGEQSGIFLDTLLEFAEDETMEIKITKEQIKGLVVD 300

Ptub FFSAGTDSTAVATEWALAELINNPRVLQKAREEVYSVVGKDRLVDEADTQNLPYIRAIVK 360
Vuni FFSAGTDSTAVATEWALAELINNPRVLQKAREEVYSVVGKDRLVDEVDTQNLPYIRAIVK 360

Ptub ETFRMHPPLPVVKRKCVEECEINGYVIPEGALILFNVWAVGRDPKYWDKPSEFRPERFLE 420
Vuni ETFRMHPPLPVVKRKCVEECEIEGCVIPEGALILFNVWAVGRDPKYWDRPSEFRPERFLE 420

Ptub T--EGDVSPIDLRGQHFQLLPFGSGRRMCPGVNLATSGMATFLASVIQCFDLQVVGPQGQ 480
Vuni NGGEGAVGPIDLRGQHFQLLPFGSGRRMCPGVNLSTSGMATLLASVIQCFDLQVLDPQGH 480

Ptub ILKGNDAKVSMEERPGLTVPREHSLMCVPLARTSVASKLLS-  519
Vuni VLKGDDAKVSMEERAGLTVPRKHNLVCLPLAKTTLAAKLLSP  522
```

Fig. 4.6. ClustalW alignment of deduced amino-acid sequences of IFS from *Pachyrhizus* (Ptub) and *Vigna* (Vuni). The mismatched amino-acids which do not have similar properties are coloured in red; the mismatched amino-acids with similar properties are highlighted in blue; the 3 gaps discovered are indicated in yellow.

4.2. Pilot study with IFS (CYP93C18) from *Pisum sativum* L.

4.2.1. Identification and isolation of *CYP93C18*

For the purposes of the present work, we selected one of the two known *IFS* genes from *Pisum sativum* L. – *CYP93C18*, GenBank accesion no. AF532999 (Cooper *et al*., 2005), subjected it to our methodical approach and were thus able to prove its correct function – for the first time in the literature. *CYP93C18* cDNA (1575 bp) was first discovered thanks to its up-regulation when pea pods were treated with the insect elicitor Bruchin B, and so named by Dr. David Nelson on the basis of sequence similarity to *CYP93C*s that had been characterized by that time (Cooper *et al.*, 2005).

On the basis of the known *CYP93C18* sequence, PCR was performed to identify the gene in the genomic DNA extracted from *Pisum sativum* L. First, to determine the optimal annealing temperatures, gradient PCR (annealing temperatures 50-65°C) using *CYP93C18*-specific primers (spanning the open reading frame and adapted for TOPO® cloning) was performed. The optimal annealing temperature was determined to be 58.3°C (Fig. 4.7.) and was used in all following steps. To obtain a sufficient amount of the desired blunt-ended PCR product with and without stop codon, PCR with appropriate primers and with Phusion DNA polymerase (proofreading polymerase) was subsequently carried out. The bands acquired (Fig. 4.8.) were excised from the gel after electrophoresis, purified and cloned, with the aim of performing stable and transient expression of the cloned gene in the model host plants.

The complete sequence obtained after TOPO® cloning was 98.7% identical to that published by Cooper *et al*. (2005), with the exception of 21 nucleotides of the 1662-bp long DNA sequence. Moreover, the amplified gene contained an 87-bp intron localized between nucleotides 903 and 991 (Fig. 4.9.). These differences resulted in the divergence of nine amino-acids in the 524-bp long deduced protein (Fig. 4.10.). None of the mismatches discovered were localized at the positions essential for the catalytic function of the protein (Sawada and Ayabe, 2005). Seven of the amino-acid mismatches could be considered as unimportant due to the similarity in the chemical properties of the amino-acid pairs (Tyr99/143 instead of Ser99/143, Lys155/158 instead of Arg155/158, Leu10 and Ile163 instead of Met10/163, Asn165 instead of Gln165). In the two remaining cases – Val122 instead of Asp122 and Gly177 instead of Glu177 – the mismatch could be potentially more serious, as it might affect intramolecular interactions in the folded protein and thus its correct catalytic function. However, these

misgivings were not fulfilled, as proved at the metabolite level (as will be explained later).

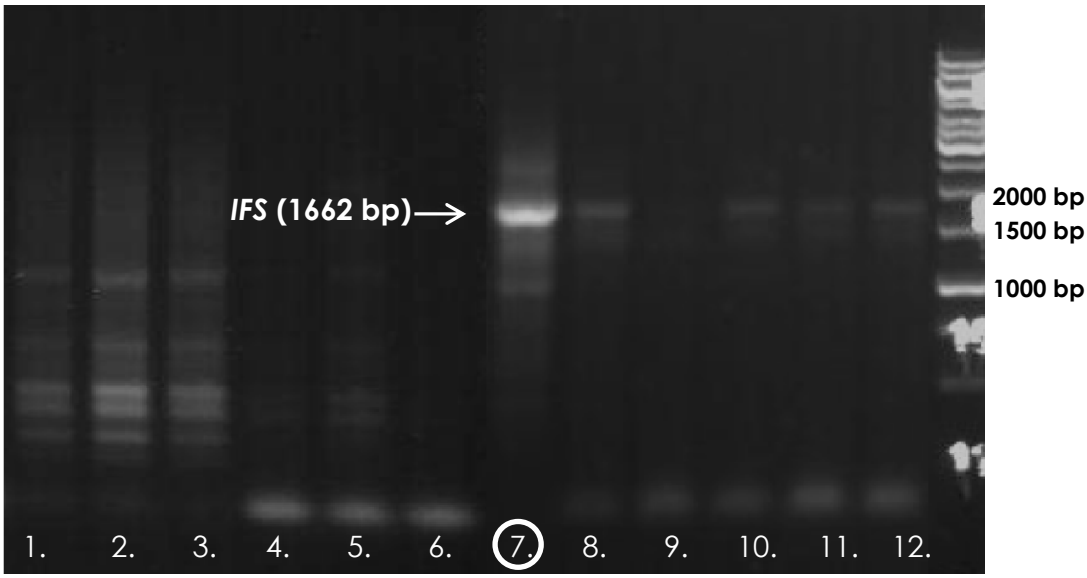


Fig. 4.7. Electrophoretogram of PCR products of the desired length of ~1700 bp obtained using *CYP93C18*-specific primers (with stop codon) and Taq DNA polymerase; The gradient of annealing temperatures was as follows: (1) 49.8 °C, (2) 50.2°C, (3) 51.1°C, (4) 52.5°C, (5) 54.3°C, (6) 56.2°C, (7) 58.3°C, (8) 60.2°C, (9) 62 °C, (10) 63.5°C, (11) 64.6°C, (12) 65.1°C. 25 µl of PCR mixture with 3 µl loading buffer were loaded per lane. The optimal temperature was found to be the 7th of the gradient used (58.3°C).

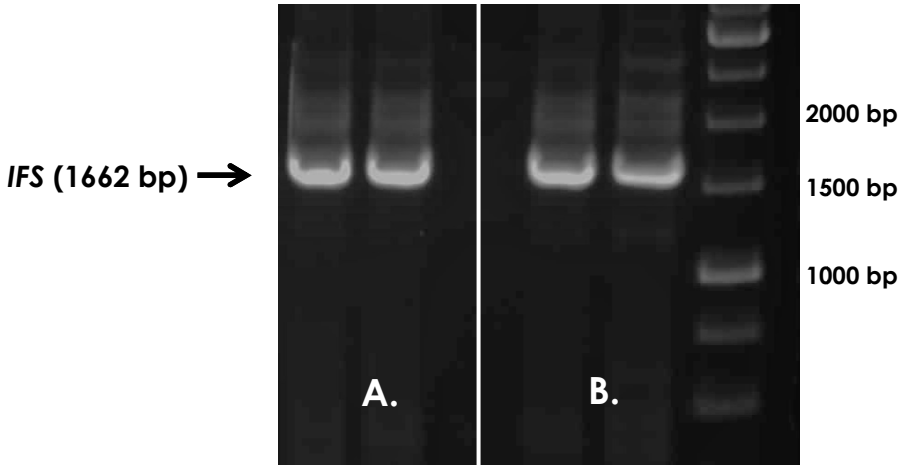


Fig. 4.8. Electrophoretogram of PCR products of desired lenght of ~1700 bp obtained using *CYP93C18*-specific primers with (A) and without (B) STOP codon and Phusion DNA polymerase in both cases. The annealing temperature of 58.3°C (+3°C according to Finzymes´s instruction) was applied. The concentration of the excised DNA was ca. 60 ng/µl in both cases.

```
Picman ATGTTGGTTGAACTTGCACTTGCTTTATTGGTGATTGCTCTATTTATACACTTGCGTCCAACACCCACTGCTAAATCAAAGGCACTTCGCCATCTTCCTA 100
Cooper ATGTTGGTTGAACTTGCACTTGCTTTAATGGTGATTGCTCTATTTATACACTTGCGTCCAACACCCACCGCTAAATCAAAGGCACTTCGCCATCTTCCTA 100

Picman ATCCACCATGTCCTAAACCTCGTCTTCCTTTTATTGGTCATCTTCATCTTTTGGATAATCCTCTTCTTCATCATTCTCTCATCCGTCTTGGAGAACGTTA 200
Cooper ATCCACCATGTCCTAAACCACGCCTTCCTTTCATTGGTCATCTTCATCTTTTGGATAATCCTCTTCTTCATCATTCTCTCATCCGTCTTGGAGAACGTTA 200

Picman TGGCCCTTTGTACTCTCTTTACTTTGGCTCCATGCCCACCATTGTTGTATCCACTCCTGATCTCTTCAAACTCTTCCTTCAAACTCATGAAGCGACTTCT 300
Cooper TGGCCCTTTGTACTCTCTTTACTTCGGCTCCATGCCCACCATTGTTGTATCCACTCCCGATCTCTTCAAACTCTTCCTTCAAACTCATGAAGCGTCATCT 300

Picman TTCAATACAAGGTTTCAAACCTCTGCTATCAGACGGTTAACTTATGATAACTCCGTTGCAATGGTTCCATTTGGACCTTACTGGAAGTTCATTAGAAAGC 400
Cooper TTCAATACAAGGTTTCAAACCTCTGCTATCAGACGGTTAACTTATGATAACTCCGTTGCAATGGATCCATTTGGACCTTACTGGAAGTTCATTAGAAAGC 400

Picman TCATCATGAATGATCTCTTTAACGCCACCACCATCAACAAGTTGAGACCCTTGAGGACTAAAGAAATCCGCAAGGTTCTTAAGGTTATTGCTAATAGCGC 500
Cooper TCATCATGAATGACCTCTTTAACGCCAGCACCATCAACAAGTTGAGACCCTTGAGGACTCGAGAAATCCGCAGAGTTCTTAAGGTTATGGCTCAGAGCGC 500

Picman TGAAACTCAAGAGCCACTTAATGTCACTGGGGAACTTCTCAAGTGGACAAACAACACAATCTCTACAATGATGTTGGGTGAGGCTGAAGAGGTTAGAGAT 600
Cooper TGAAACTCAAGAGCCACTTAATGTCACTGAGGAGCTTCTCAAGTGGACAAACAACACAATCTCTACAATGATGTTGGGTGAGGCTGAAGAGGTTAGAGAT 600

Picman ATTGCTCGTGATGTTCTTAAGATCTTTGGGGAATACAGTCTCACCGACTTCATTTGGCCTTTGAAGATGTTTAAGTTTGGGAACTATGAGAAGAGAACTG 700
Cooper ATTGCTCGTGATGTTCTTAAGATCTTTGGGGAATACAGTCTCACCGACTTCATTTGGCCTTTGAAGATGTTTAAGTTTGGGAACTATGAGAAGAGAACTG 700

Picman AAGCCATTTTCAACAAATATGATCCTATCATTGAAAAAGTTATCAAGAAAAGACAAGAGATTGTGAATAAAAGAAAAGAGAAAAATGGAGAAATCCAAGA 800
Cooper AAGCCATTTTCAACAAATATGATCCTATCATTGAAAAAGTTATCAAGAAAAGACAAGAGATTGTGAATAAAAGAAAAGAGAAAAATGGAGAAATCCAAGA 800

Picman AAGTGAGCAAAGTGTAGTTTTTCTTGATACTTTGCTTGAATTTGCTCAAGATGAGACAATGGAGATCAAAATTACAAAGGAACAAATCAAGGGTCTTGTT 900
Cooper AAGTGAGCAAAGTGTAGTTTTTCTTGATACTTTGCTTGAATTTGCTCAAGATGAGACAATGGAGATCAAAATTACAAAGGAACAAATCAAGGGTCTTGTT 900

Picman GTGGTGAGTTTCTTTTTCATCTAGTTGCTTTGTTATTATTATATTATAGATAATAATAAAAGATTGTCTTTCTCTCTCTATGATATGCAGGATTTCTTCT…1000
Cooper GTG----------------------------------------------------------------------------------GATTTCTTCT…1000
```

Fig. 4.9. ClustalW alignment of the first 1000 nucleotides of our and of Cooper´s *CYP93C18* sequences, respectively. The remaining parts of these sequences were 100% identical. The 21 discovered mismatches are coloured in red; the 87 nucleotides long intron is highlighted in yellow.

```
Picman MLVELALALLVIALFIHLRPTPTAKSKALRHLPNPPCPKPRLPFIGHLHLLDNPLLHHSLIRLGERYGPLYSLYFGSMPTIVVSTPDLFKLFLQTHEATS 100
Cooper MLVELALALMVIALFIHLRPTPTAKSKALRHLPNPPCPKPRLPFIGHLHLLDNPLLHHSLIRLGERYGPLYSLYFGSMPTIVVSTPDLFKLFLQTHEASS 100

Picman FNTRFQTSAIRRLTYDNSVAMVPFGPYWKFIRKLIMNDLFNATTINKLRPLRTKEIRKVLKVIANSAETQEPLNVTGELLKWTNNTISTMMLGEAEEVRD 200
Cooper FNTRFQTSAIRRLTYDNSVAMDPFGPYWKFIRKLIMNDLFNASTINKLRPLRTREIRRVLKVMAQSAETQEPLNVTEELLKWTNNTISTMMLGEAEEVRD 200

Picman IARDVLKIFGEYSLTDFIWPLKMFKFGNYEKRTEAIFNKYDPIIEKVIKKRQEIVNKRKEKNGEIQESEQSVVFLDTLLEFAQDETMEIKITKEQIKGLV 300
Cooper IARDVLKIFGEYSLTDFIWPLKMFKFGNYEKRTEAIFNKYDPIIEKVIKKRQEIVNKRKEKNGEIQESEQSVVFLDTLLEFAQDETMEIKITKEQIKGLV 300

Picman VDFFSAGTDSTAVATEWTLAELINNPRVLKKAREEIDSVIGKDRLVDESDVQNLPYIRAMVKEVFRMHPPLPVVKRKCTEECEINGYVIPEGALVLFNVW 400
Cooper VDFFSAGTDSTAVATEWTLAELINNPRVLKKAREEIDSVIGKDRLVDESDVQNLPYIRAMVKEVFRMHPPLPVVKRKCTEECEINGYVIPEGALVLFNVW 400

Picman AVGRDPKYWKRPLEFRPERFLENAGEGEAGSVDLRGQHFQLLPFGSGRRMCPGVNLATAGMATLLASIIQCFDLQVPGPDGKILKGDDAKVSMKERAGLS 500
Cooper AVGRDPKYWKRPLEFRPERFLENAGEGEAGSVDLRGQHFQLLPFGSGRRMCPGVNLATAGMATLLASIIQCFDLQVPGPDGKILKGDDAKVSMKERAGLS 500

Picman VPRAQNLVCVPLARDGLAAKLLSS* 524
Cooper VPRAQNLVCVPLARDGLAAKLLSS* 524
```

Fig. 4.10. ClustalW alignment of deduced amino-acid sequences of our and of Cooper´s CYP93C18, respectively. The nine discovered mismatches are coloured in red; the three amino-acid residues essential for aryl migration (Sawada and Ayabe, 2005) are highlighted in blue.

4.2.2. TOPO® cloning of *CYP93C18*

According to the Gateway® cloning manual, both blunt-ended PCR products with and without stop codon were separately introduced into pENTR™/D-TOPO® to obtain the entry clones (Fig. 4.11.), as described in section 3.4.6. The following day after heat shock transformation of *E.coli* TOP 10 competent cells, tens of white colonies appeared on the selective plates with 50 µg/ml kanamycin (Fig. 4.12.). The colony PCR with randomly-chosen four colonies as DNA teplate and *CYP93C18*-specific primers were then carried out to confirm the presence of the gene of interest (Fig. 4.13.). One of the successfully-transformed colonies in both cases was cultivated overnight and pENTR/D-TOPO::IFS constructs were isolated using GeneJET™ Plasmid Miniprep Kit. The concentration of the plasmids acquired was determined by means of NanoDrop to be ca. 250 ng/µl in both cases. The final PCR with the plasmids diluted to a concentration of ca. 25 ng/µl, was then performed. *CYP93C18*-specific primers were used in order to verify the **presence** of the insert in the vector; cross combination of *CYP93C18*-specific primer Forward and primer M13 Reverse (pENTR/D-TOPO-specific, 157 bp downstream from the insert) was used to confirm the **correct orientation** of the insert in the vector (Fig. 4.14.).

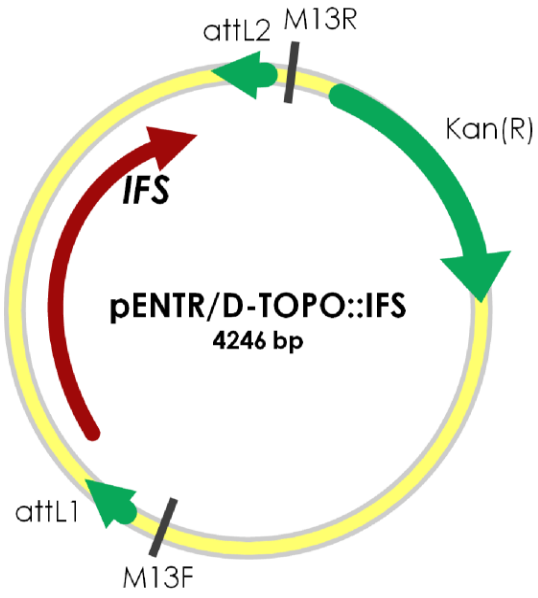


Fig. 4.11. The entry clone with inserted *CYP93C18* (*IFS*), which is the same in the case of the insert with and without stop codon. The simplified schematic layout of the clone was created according to the *in silico* cloning generated by the Vector NTI Suite 9.0.0.

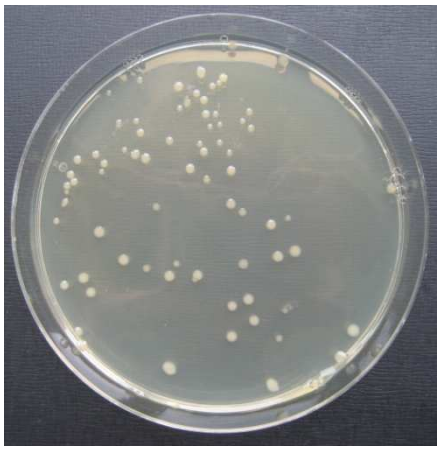


Fig. 4.12. Selective plate with well-spaced *E.coli* colonies carrying the introduced entry clone (pENTR/D-TOPO::IFS+stop). The plate was very similar in appearance to those with colonies carrying *IFS –stop*.

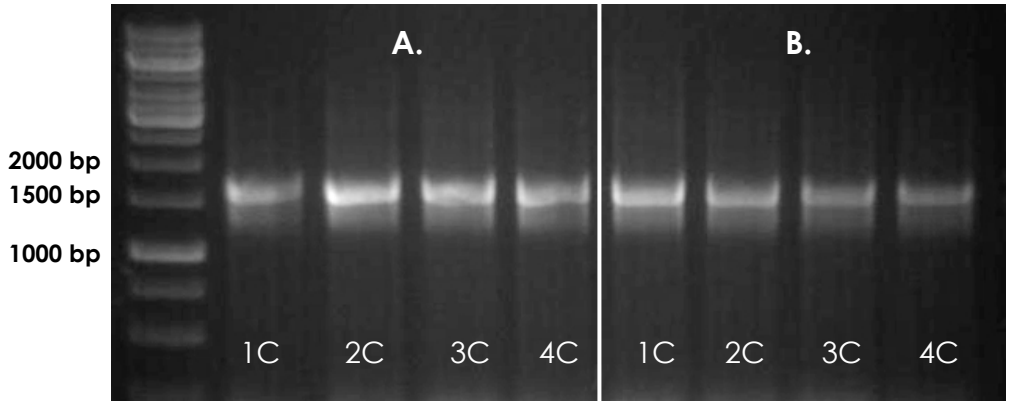


Fig. 4.13. Electrophoretogram of the colony PCR products confirming the presence of *CYP93C18* insert (~1700 bp) in four selected colonies. (A) Insert with stop codon, (B) insert without stop codon. (1-4C) Colony number.

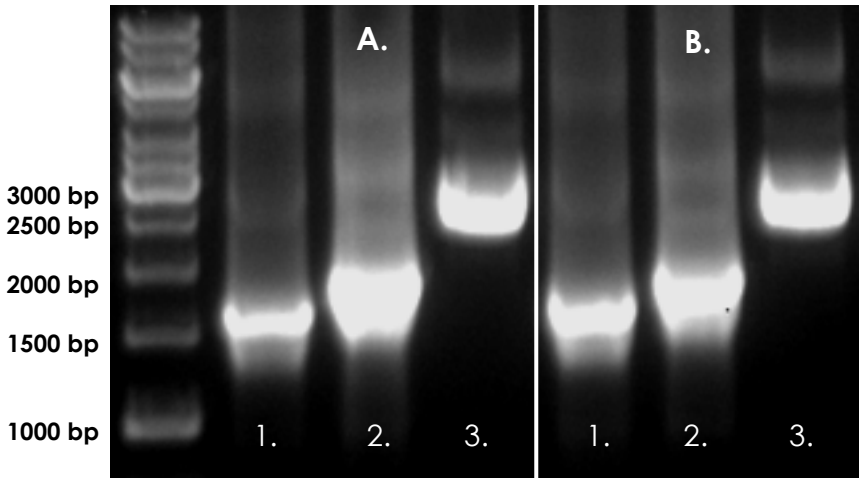


Fig. 4.14. Electrophoretogram confirming the presence and the correct orientation of the insert in the isolated plasmids. (A) Insert with stop codon, (B) insert without stop codon. (1) PCR product (~1700 bp) obtained using *CYP93C18*-specific primers, (2) PCR product (~1900 bp) obtained using cross combination of *CYP93C18*-specific primer F and M13 R, (3) the isolated plasmid with the inserted *CYP93C18* itself (~3000 bp; 5 µl with 3 µl of loading buffer loaded per lane).

4.2.3. Construction of *CYP93C18* expression clone for stable expression

In order to secure the stable expression of *CYP93C18* (with stop codon) under CaMV35S promoter, LR recombination between relevant entry clone (pENTR/D-TOPO::IFS+stop) and destination vector pGWB2 was performed. The final construct obtained (Fig. 4.15.) was introduced into *Alpha*-Select Gold Efficiency competent cells. As expected, dozens of white colonies appeared on the selective plates containing 50 µg/ml kanamycin and 50 µg/ml hygromycin (Fig. 4.16.). As in the case of section 4.2.2., colony PCR followed, with randomly-chosen five colonies as DNA template and *CYP93C18*-specific primers (Fig. 4.17.). Two of the successfully transformed colonies were cultivated overnight and pGWB2::IFS constructs were isolated using GeneJET™ Plasmid Miniprep Kit. The concentration of acquired plasmids was determined by means of NanoDrop as ca. 40 ng/µl. The control PCR with the plasmids diluted to the final concentration of ca. 20 ng/µl was then perfomed. *CYP93C18*-specific primers were used in order to verify the presence of the insert in the vector; cross combination of *CYP93C18*-specific Forward and pGWB2 Reverse (pGWB2-specific, 55 bp downstream from the insert) primers was used to confirm the correct orientation of the insert in the vector (Fig. 4.18.).

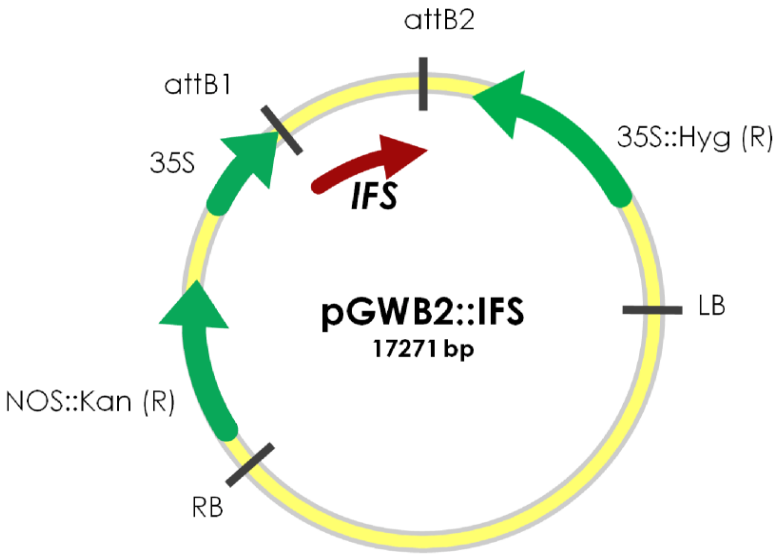


Fig. 4.15. The expression clone with inserted *CYP93C18* (*IFS+stop*). The simplified schematic layout of the expression clone was created according to that generated *in silico* by the Vector NTI Suite 9.0.0.

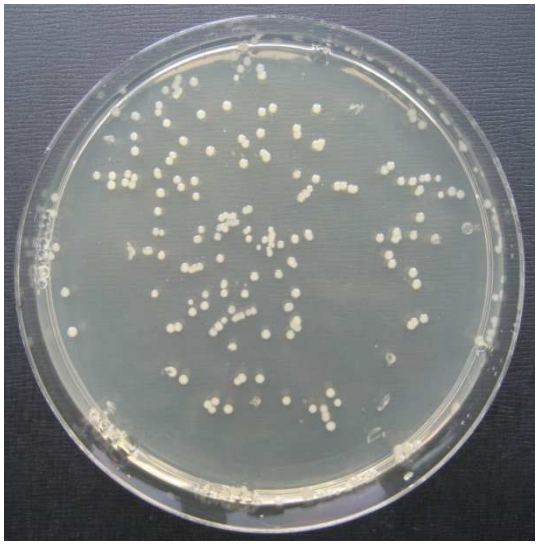


Fig. 4.16. Selective plate with well-spaced *Alpha*-Select Gold colonies carrying introduced expression clone pGWB2::IFS+stop. The plate was very similar in appearance to those with colonies carrying expression clone pGWB5::IFS – stop.

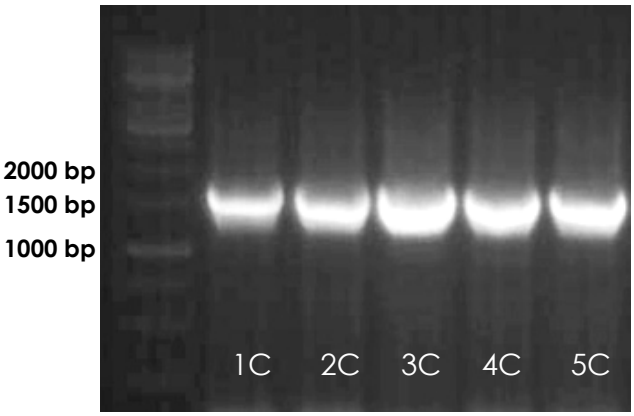


Fig. 4.17. Electrophoretogram of the colony PCR products verifying the presence of *CYP93C18* insert (~1700 bp) in five selected colonies. (1-5C) Colony number.

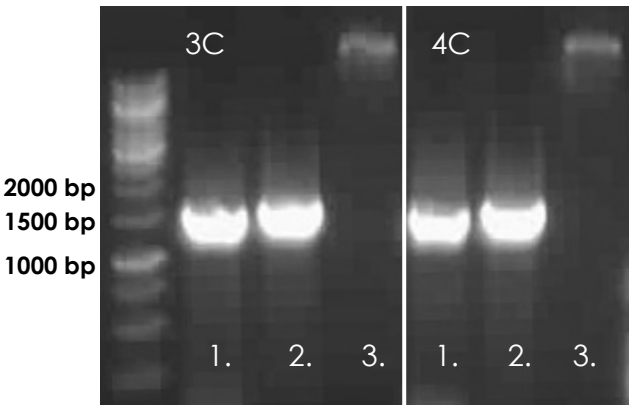


Fig. 4.18. Electrophoretogram confirming the presence and the correct orientation of the insert in the plasmids isolated from colonies 3C and 4C (1) PCR product (~1700 bp) obtained with *CYP93C18*-specific primers, (2) PCR product (≥1700 bp) obtained with the cross combination of *CYP93C18*-specific F and pGWB2 R primers (3) the isolated plasmid with the inserted *CYP93C18* itself (~ 17,000 bp; 5 µl with 3 µl of loading buffer loaded per lane).

4.2.4. Construction of *CYP93C18* expression clone for transient expression

The procedure used was the same as that described in section 4.2.3. The destination vector pGWB5 was used to secure the IFS over-expression under CaMV35S promoter and to enable C-terminal phusion of *IFS –stop* with GFP (Fig. 4.19.). The colony PCR (Fig. 4.20.), plasmid isolation and the final control PCR (Fig. 4.21.) were carried out in the same manner as previously described.

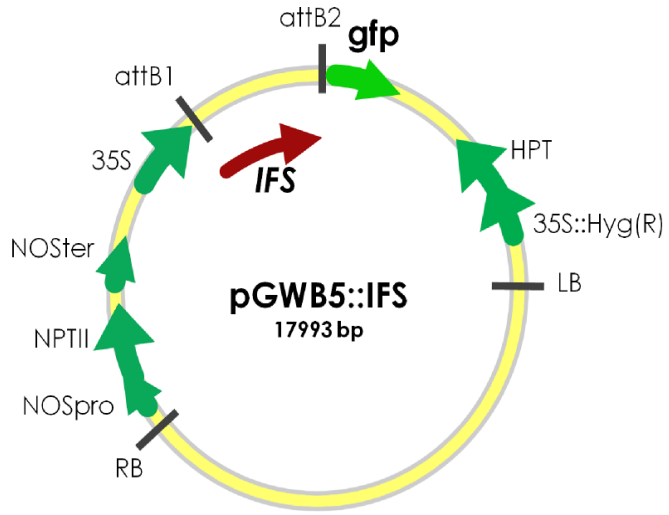


Fig. 4.19. The expression clone with inserted *CYP93C18* (*IFS –stop*). According to the Vector NTI Suite 9.0.0.

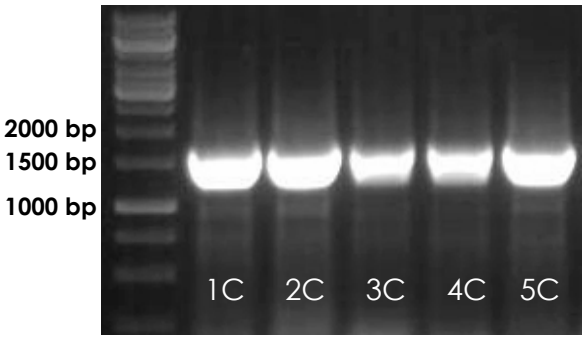


Fig. 4.20. Electrophoretogram of the colony PCR products verifying the presence of *CYP93C18* insert (~1700 bp) in five selected colonies. (1-5C) Colony number.

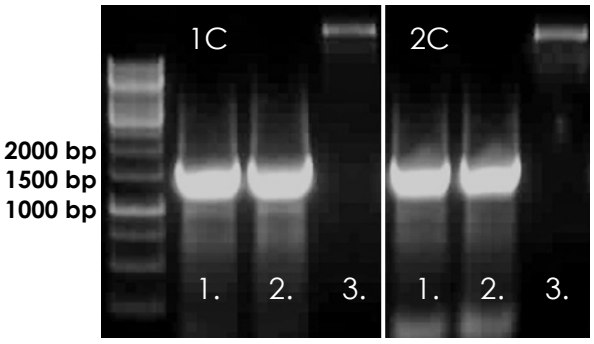


Fig. 4.21. Electrophoretogram confirming the presence and the correct orientation of the insert in the plasmids isolated from colonies 1C and 2C (1) PCR product (~1700 bp) obtained using *CYP93C18*-specific primers, (2) PCR product (≥1700 bp) obtained using cross combination of *CYP93C18*-specific primer F and pGWB R, (3) the isolated plasmid with the inserted *CYP93C18* itself (~ 17,000 bp; 5 µl with 3 µl of loading buffer loaded per lane).

4.2.5. Transformation of *Agrobacterium tumaefaciens*

The next logical step of our effort to over-express *CYP93C18*, was the introduction of the obtained expression clones into "a natural genetic engineer" *Agrobacterium* which possesses the ability to tranfer a gene of interest with a selectable marker into a plant genome. The isolated plasmids pGWB2::IFS+stop and pGWB5::IFS–stop were separately transformed into *Agrobacterium* GV3101 by means of electroporation. Transformants were then selected on the YEB-plates containing 50 µg/ml kanamycin, 50 µg/ml hygromycin, 50 µg/ml gentamycin and 100 µg/ml rifampicin (Fig. 4.22.). Randomly-chosen colonies were checked by colony PCR with appropriate *CYP93C18*-specific primers in both cases (Fig. 4.23.).

Fig. 4.22. Selective plate with transformed *Agrobacterium* colonies (slightly over-grown) carrying introduced expression clone pGWB2::IFS+stop. The plate was very similar in appearance to those with colonies carrying expression clone pGWB5::IFS –stop.

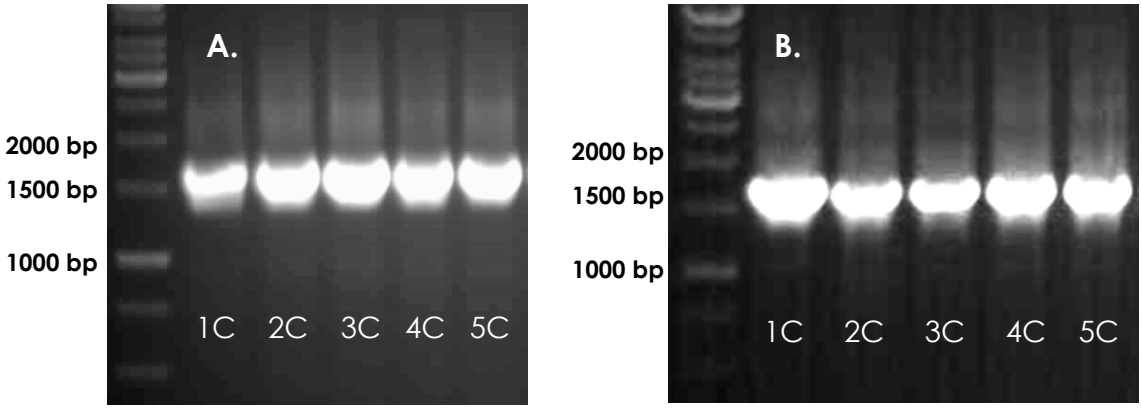


Fig. 4.23. Electrophoretogram of the *Agrobacterium* colony PCR products confirming the presence of *CYP93C18* insert (~1700 bp) in five selected colonies. (1-5C) Colony number. (A) pGWB2::IFS, (B) pGWB5::IFS.

4.2.6. *Arabidopsis thaliana* transformation with pGWB2::IFS

After the construction of the plant expression vector pGWB2::IFS, the gene of interest (under the 35S promoter) was introduced via *Agrobacterium tumaefaciens* into *Arabidopsis thaliana*, using the standardized floral dip method. Following the infiltration, transformed plants matured in the growth chamber. Their seeds were harvested, sterilized and sown individually on ten MS-plates with 50 µg/ml hygromycin. As on average only one out of 200 seeds sown on the plates survived the selection, transformation efficiency was stated to be 0.5% (Fig. 4.24.). Thus, only 10 putative transformed *Arabidopsis* seedlings were obtained. They were subsequently transplanted into soil (Jiffy peat pot), grown under standard conditions and analyzed (see section 4.2.7.). The above-mentioned procedure was repeated to obtain homozygous lines of transformants in T2 generation. The seeds of T2 generation originating from individual T1 plants were grown on plates separately and homozygous and heterozygous lines were thus simply distinguishable: all seedlings appeared on the plate in the case of the stable homozygous line; approximately ¼ of the seeds did not survive in the case of heterozygous lines, due to the resistance marker segregation (Fig. 4.25.). It should also be mentioned that transgenic *Arabidopsis* displayed no phenotypical changes in comparison with wild-type *Arabidopsis* (Fig. 4.26.).

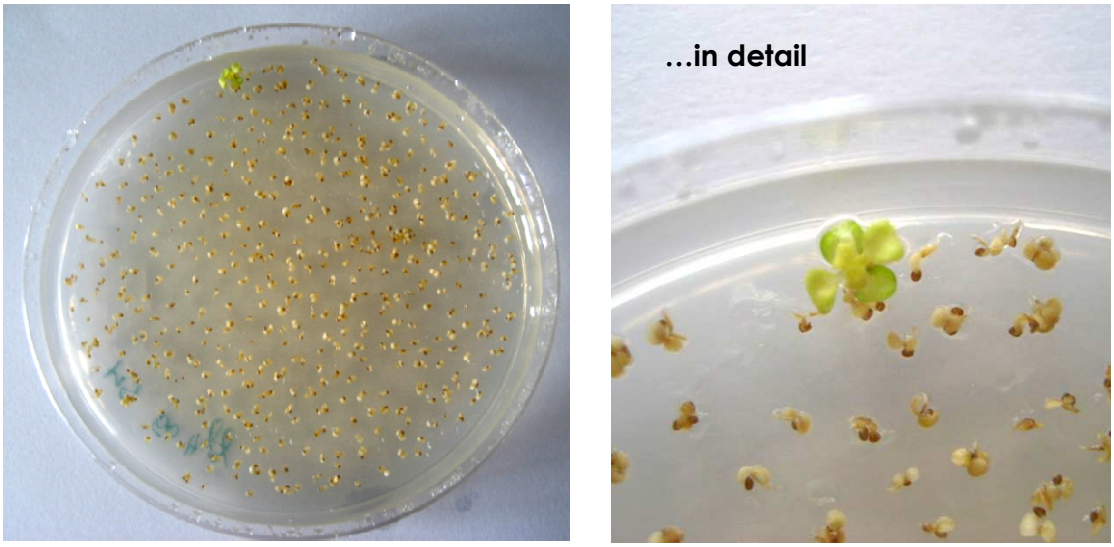


Fig. 4.24. Selection of seeds from transgenic *Arabidopsis* on MS-plate. On average, only 0.5% seeds survived the selection.

Fig. 4.25. Selected seedlings of T2 generation. (A) Homozygous line (all seeds germinated); (B) Heterozygous line (¼ non-germinated seeds).

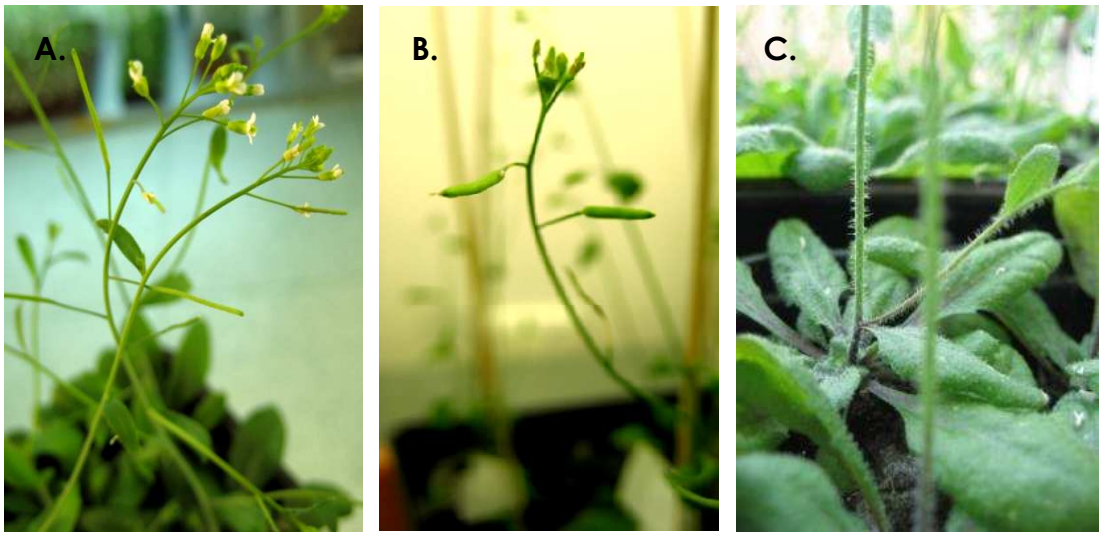


Fig. 4.26. Wild-type *Arabidopsis* plant (A) compared to the T1 generation of transgenic *Arabidopsis* plants overexpressing CYP93C18 (B, C). No apparent phenotype changes were observed.

4.2.7. Verification of correct function of CYP93C18 transgene

Transgenic hygromycin-resistant plants and their next generation were then analysed at four different levels: DNA, RNA, proteins and metabolites.

4.2.7.1. DNA level

DNA isolated from all transgenic plants of T1 and 20 randomly-chosen plants of T2 generation was analysed by PCR with *CYP93C18*-specific primers to verify the presence of the transgene. *CYP93C18* was found to be present in all T1 *Arabidopsis* plants (Fig. 4.27.) and in most of the individuals of T2 generation (Fig. 4.28.A). The corresponding band was also obtained in *Pisum* – as a positive control (Fig. 4.28.B), but not in wild-type *Arabidopsis* – the negative control (Fig. 4.28.C).

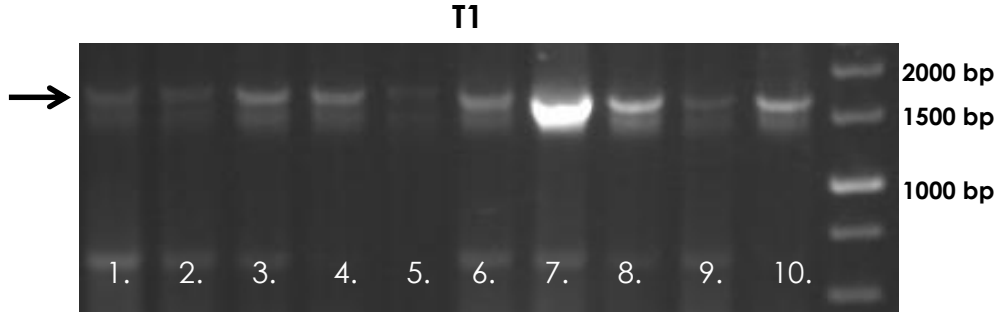


Fig. 4.27. Ten transgenic *Arabidopsis* plants of T1 generation. Electrophoretogram confirming the presence of *CYP93C18* (arrowhead shows *IFS* 1662 bp long).

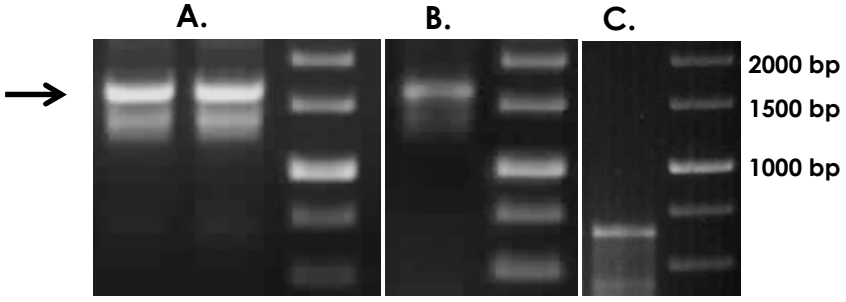


Fig. 4.28. Electrophoretogram of PCR products confirming the presence of *CYP93C18* (arrowhead shows *IFS* 1662 bp long). (A) Two chosen PCR products from T2 homozygous line of transgenic *Arabidopsis*; (B) *Pisum* as a positive control; (C) wild-type *Arabidopsis* as a negative control.

4.2.7.2. RNA level

RNA was isolated from T2 *Arabidopsis* leaves and analysed by RT-PCR in order to verify the presence of *CYP93C18* mRNA, and thus the active transcription of the transgene, even without any previous stress stimulation. The same procedure was carried out in *Pisum* and wild-type *Arabidopsis* as controls. RNA and cDNA yields (measured by NanoDrop) were as follows:

Sample	*RNA* (ng/µL)	*cDNA* (ng/µL)
Transformed *Arabidopsis* 1	420	984
Transformed *Arabidopsis* 2	300	898
Wild-type *Arabidopsis* 1	21	1171
Wild-type *Arabidopsis* 2	92	1593
Pisum 1	3354	1362
Pisum 2	1721	1232

The results obtained proved that *CYP93C18* mRNA was present in transgenic *Arabidopsis* and *Pisum* but not in wild-type *Arabidopsis* (Fig. 4.29.)

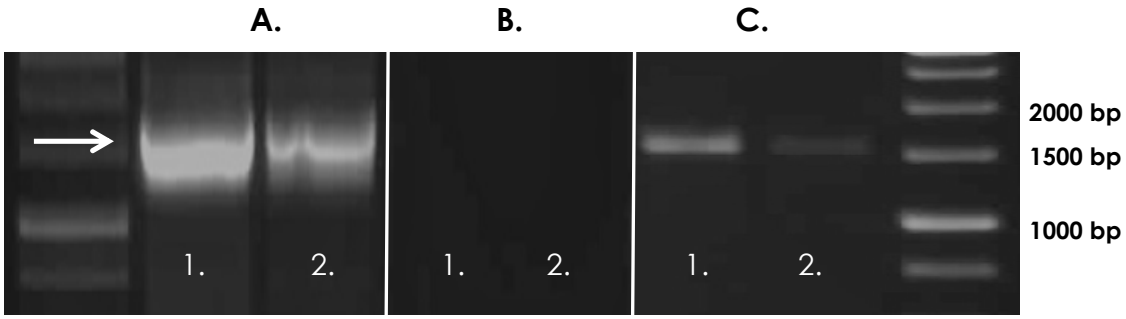


Fig. 4.29. Electrophoretogram of two selected PCR products obtained by PCR with cDNA and *CYP93C18*-specific primers (arrowhead shows *IFS* without intron 1575-bp long). (A) T2 generation of transgenic *Arabidopsis*; (B) wild-type *Arabidopsis* as a negative control; (C) *Pisum* as a positive control.

4.2.7.3. Protein level

To confirm the correct expression of the CYP93C18, total protein extraction, accurate quantification and subsequent immunoblotting with an IFS-specific peptide antibody (designed accordingly to Yu *et al.*, 2000; Fig. 4.31.) were performed. 1-D SDS-PAGE western blot of 10 µg total protein extracted from transgenic and wild-type *Arabidopsis* and *Pisum* was reversibly stained with Ponceau S (Fig. 4.30.) for control detection of separated proteins. In contrast to Yu´s results, the subsequent immunological detection showed that the antibody reacted in a non-specific manner with the total protein extracted from the plants examined, and, accordingly, we noted the concomitant failure of the discrete IFS band to appear sufficiently distinctly in the immunoblot (Fig. 4.32.). In spite of this, the CYP93C18 protein (59.4 kDa) may be assumed to be present in transgenic *Arabidopsis* and *Pisum*, but not in wild-type *Arabidopsis*. A similar problem due to the non-specific binding of the IFS antibody has already been described in the literature, once (Jaganath, 2005).

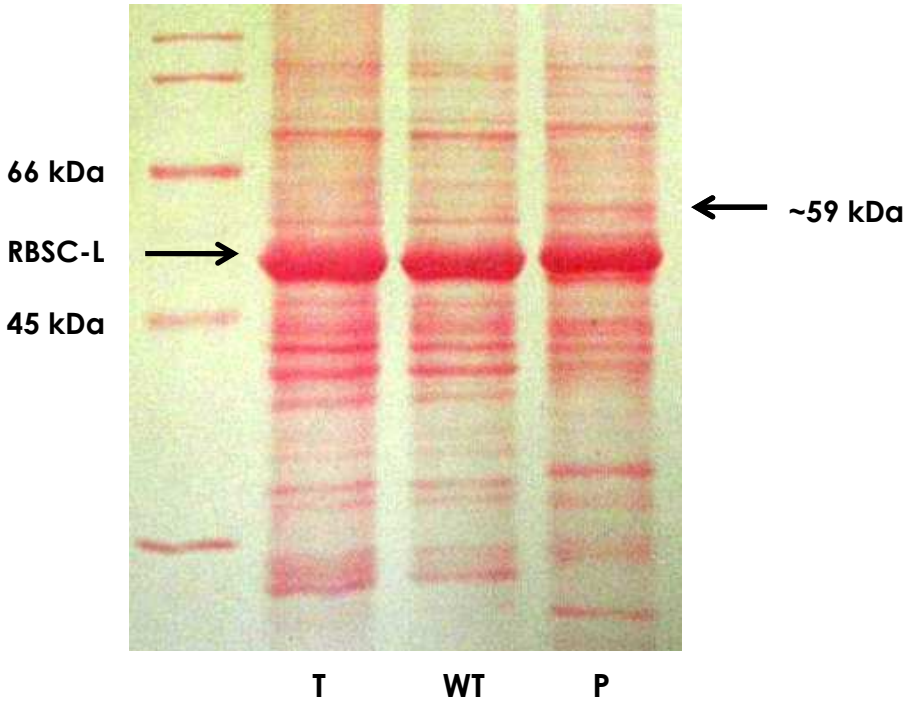


Fig. 4.30. 1-D SDS-PAGE western blot of total protein stained with Ponceau S. Rubisco large subunit (RBSC-L; 55 kDa) occupies the major proportion of the proteins extracted. IFS of the presumed size 59.4 kDa is present approximately halfway between RBSC-L and the 66 kDa marker. (T) Transgenic *Arabidopsis*; (WT) wild-type *Arabidopsis*; (P) *Pisum*.

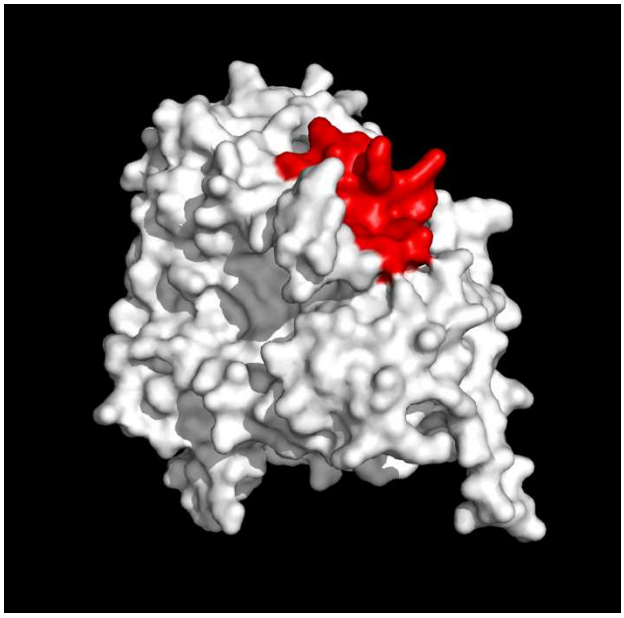


Fig. 4.31. The modelled CYP93C18, with the red region indicating the epitop of the peptide NH_2-DPKYWKRPLEFRPER. This peptide, used for IFS-specific antibody production (conjugated with KLH), is localized in a highly-accessible site of CYP93C18.

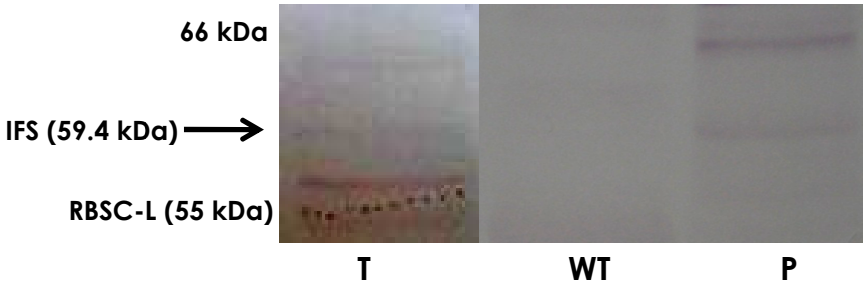


Fig. 4.32. 1-D SDS-PAGE immunoblot of the total protein of the plants examined. Although the bands are poorly visible, the CYP93C18 protein (59.4 kDa) may be assumed to be present in transgenic *Arabidopsis* and *Pisum* but not in wild-type *Arabidopsis*; the arrowhead shows a protein of size ca. 59 kDa – putative isoflavone synthase. (T) Transgenic *Arabidopsis*; (WT) wild-type *Arabidopsis*; (P) *Pisum*.

4.2.7.4. Metabolite level

HPLC-MS analysis of extracts from whole shoots and seeds of the plants examined revealed the main indicator of the correct function of the introduced CYP93C18: the presence of **isoflavonoids** in the transgenic *Arabidopsis*, despite the absence of previous stress induction. Using 15 different isoflavonoid standards (Fig. 4.34.), we detected the isoflavone genistein and its methylated derivative, tectorigenin (4´,5,7-trihydroxy-6-methoxyisoflavone), in the shoots and seeds of the transgenic *Arabidopsis*, respectively (Fig. 4.35. A and B). A positive control – *Pisum* contained 5 different forms of isoflavonoids in total (Fig. 4.35. E and F). None of the isoflavonoids on which we focused were present in the shoots or seeds of the wild-type *Arabidopsis* (Fig. 4.35. C and D). The chemical structures of the isoflavonoids detected are shown in Fig. 4.33. The quantities of the isoflavonoids measured were as follows:

Sample	***Content of isoflavonoids***	
	***Shoot* (µg/g dry weight)**	***Seeds* (µg/g dry weight)**
Transformed *Arabidopsis*	1.58 tectorigenin	0.5 genistein
Wild-type *Arabidopsis*	none	none
Pisum	none daidzein 0.31daidzin 0 genistein 0.35 prunetin 0.41 puerarin	0.55 daidzein 6.96 daidzin 0.33 genistein 0.14 prunetin none puerarin

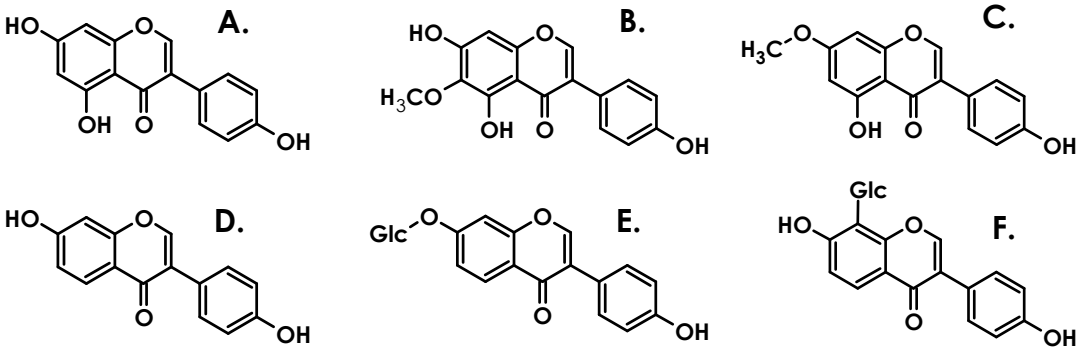


Fig. 4.33. Chemical structures of the detected isoflavonoids. (A) genistein; (B) tectorigenin; (C) prunetin (7-*O*-methylated genistein); (D) daidzein; (E) daidzin (7-*O*-glucoside of daidzein); (F) puerarin (8-*C*-glucoside of daidzein). Drawn in ACD/ChemSketch according to PubChem http://pubchem.ncbi.nlm.nih.gov/).

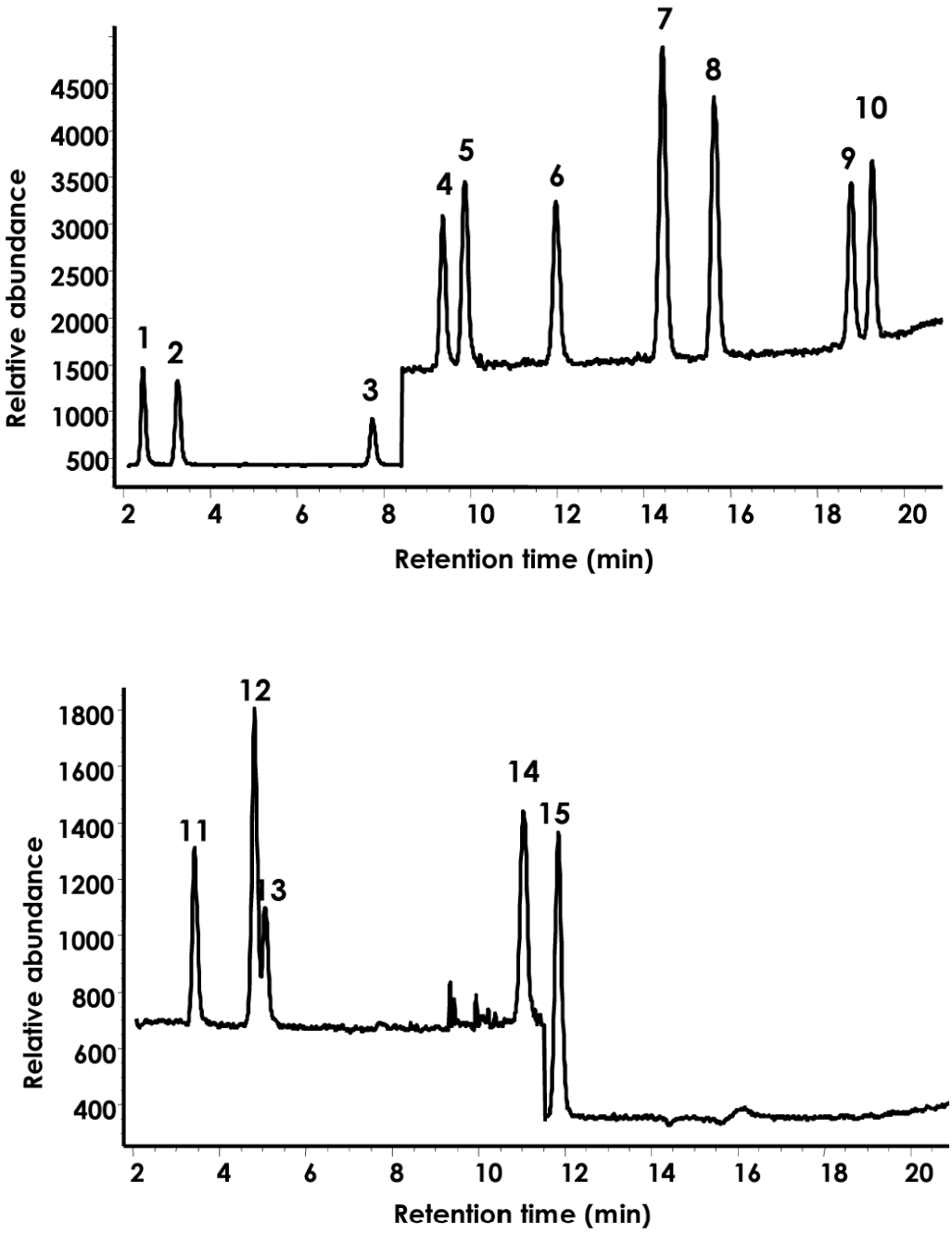


Fig. 4.34. 15 isoflavonoid standards used: 1-puerarin (r.t. 2.436), 2- daidzin (r.t. 3.235), 3-ononin (r.t. 7.739), 4-daidzein (r.t. 9.354), 5-glycitein (r.t. 9.867), 6- genistein (r.t. 11.974), 7- isoformononetin (r.t.14.429), 8-formononetin (r.t. 15.624), 9-puerarin (r.t. 17.789), 10-biochanin A (r.t. 19.270), 11-glycitin (r.t. 3.420), 12-genistin (r.t. 4.809), 13-tectoridin (r.t. 5.054), 14- sissotrin (r.t. 11.041), 15-tectorigenin (r.t. 11.840). Abbreviation: r.t. – retention time.

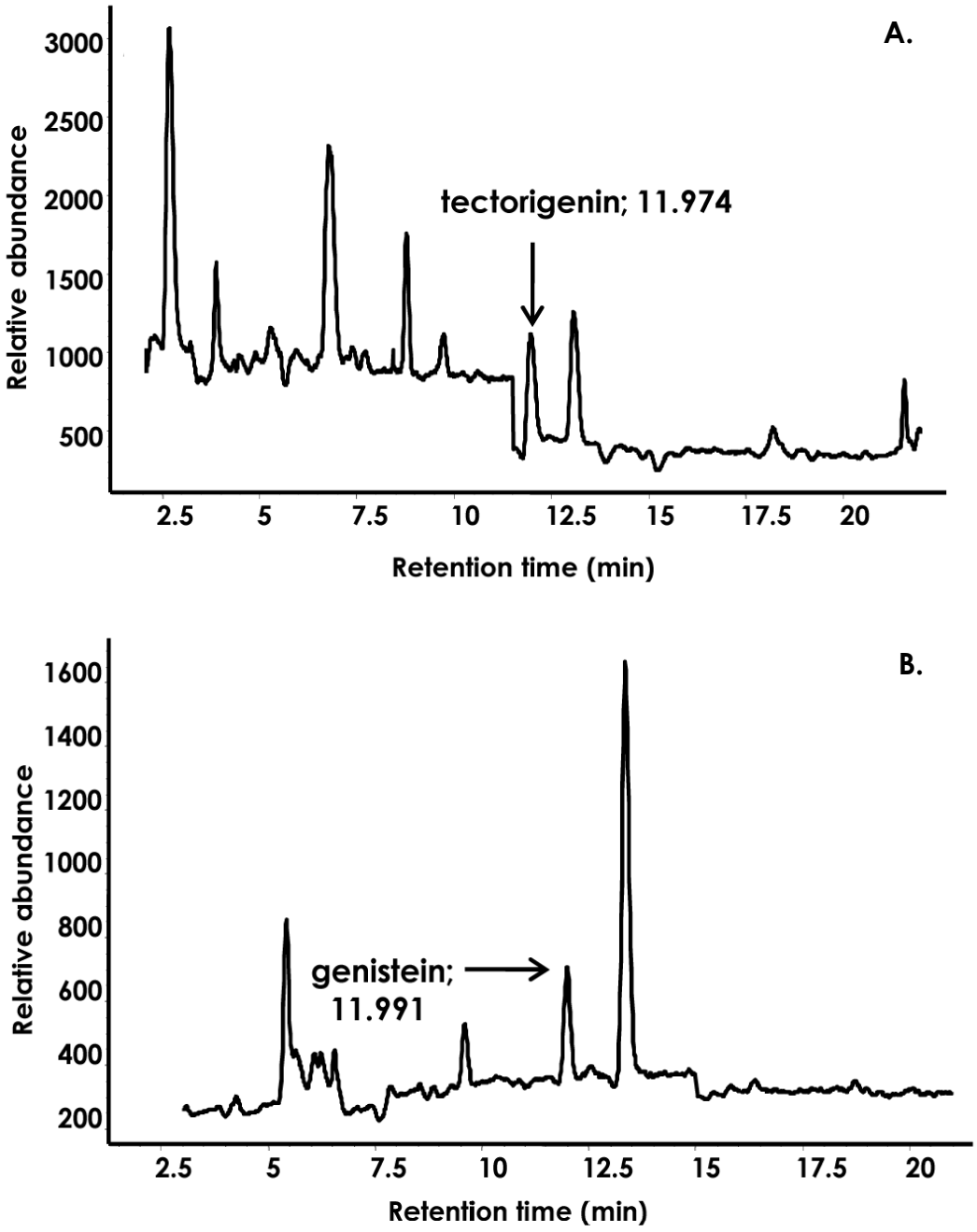
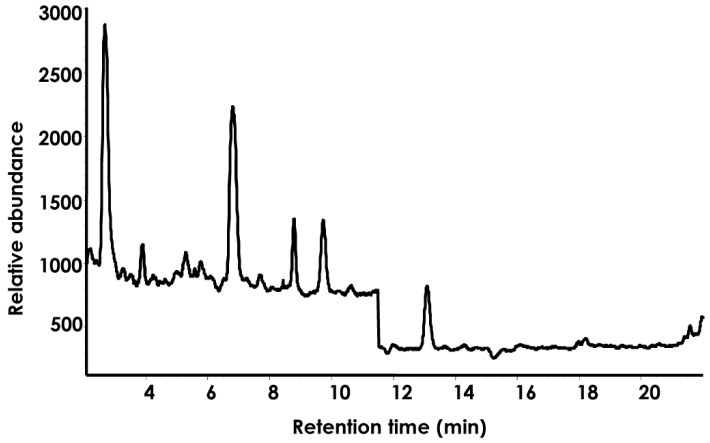
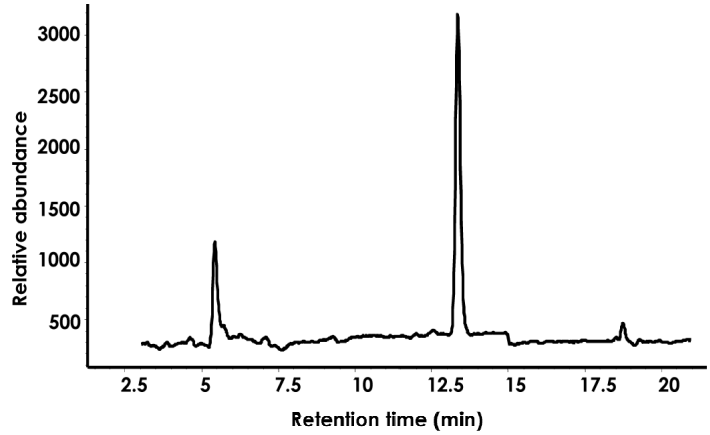


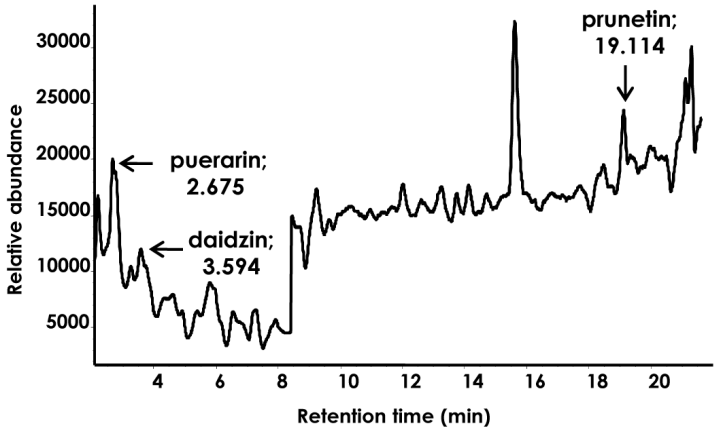
Fig. 4.35. HPLC-MS chromatograms of isoflavonoid content in the examined plants: Extracts from transgenic *Arabidopsis* shoots (A) and seeds (B). Genistein and genistein derivative tectorigenin were detected.

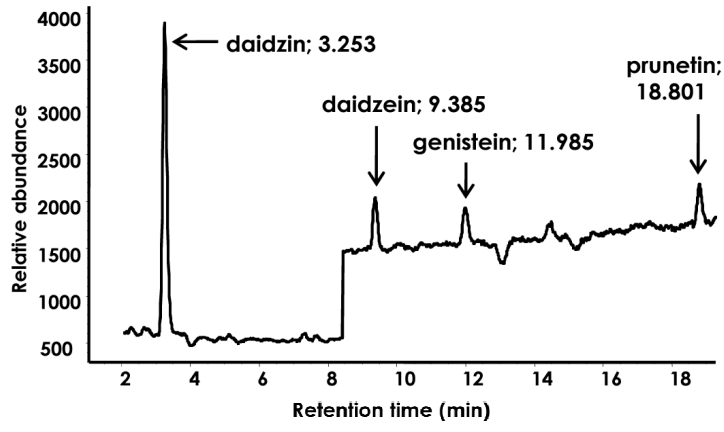
→

Next page: Extracts from wild-type *Arabidopsis* shoots (C) and seeds (D) – no isoflavonoids detected; extracts from *Pisum* shoots (E) and seeds (F) – five isoflavonoids detected.


C.
Relative abundance
3000
2500
2000
1500
1000
500
4
6
8
10
12
14
16
18
20
Retention time (min)


D.
Relative abundance
3000
2500
2000
1500
1000
500
2.5
5
7.5
10
12.5
15
17.5
20
Retention time (min)


E.
prunetin;
19.114
puerarin;
2.675
daidzin;
3.594
Relative abundance
30000
25000
20000
15000
10000
5000
4
6
8
10
12
14
16
18
20
Retention time (min)


F.
daidzin; 3.253
daidzein; 9.385
genistein; 11.985
prunetin;
18.801
Relative abundance
4000
3500
3000
2500
2000
1500
1000
500
2
4
6
8
10
12
14
16
18
Retention time (min)

4.2.8. Visualization of CYP93C18 *in vivo*

Next, our objective to visualize CYP93C18´s localization *in vivo* was met. It is frequently stated in the literature that isoflavone synthase is localized on the membrane of the endoplasmic reticulum (Kochs and Grisebach, 1984; Liu and Dixon 2001 and others). Using the on-line tool SignalP 3.0 (Bendtsen *et al*., 2004), the N-terminal signal peptide of CYP93C18 was predicted with 99.6% probability (Hidden Markov Models). The cleavage site was determined to lie between the 18th and 19th amino-acid residues, with a not very high probability of 39.2%. The prediction of ER targeting was verified by the *Agrobacterium*-mediated transient expression of 35S::CYP93C18::GFP fusion protein (construct pGWB5::IFS, see section 4.2.4.) in *Nicotiana benthamiana* leaves. The desired fluorescent signal was observed by means of a confocal microscope in the endoplasmic reticulum, as predicted (Fig. 4.36. A-D). The 35S::CYP93C18::GFP localization was identical to that of GFP::HDEL (pBIN *m-gfp5*-ER) fusion protein marker for the ER localization (Fig. 4.36. F). In contrast, these results were very different from the diffused localization of free GFP in the cytoplasm and nucleus (Fig. 4.36. E).

4.2.9. IFS association with endoplasmic reticulum membrane

The question as to how the IFS is bound to the ER membrane, however, remains to be answered. In the case of IFS, no signal anchor (i.e. uncleaved signal peptide) was recognised by the HMM, nor was any potential modification site found within the CYP93C18 sequence. However, further investigation using the on-line tools SOSUI (Mitaku Group, Department of Applied Physics, Nagoya University) and TMpred (Hofmann and Stoffel, 1993), revealed the presence of the N-terminal hydrophobic transmembrane helix with inner-outer membrane orientation, corresponding to the predicted signal peptide within the IFS sequence. We constructed a model of the CYP93C18 3D structure using the Modeller9v5 program. As templates, known 3D structures of mammalian P450 (e.g. rcsb code: 1tqn, 2hi4 and 3e6i) were utilized as they were found to be the most suitable by the Psipred threading algorithm. On the basis of this model and previous predictions, we propose a mechanism of IFS membrane-binding, with the N-terminal helix embedded in a membrane (Fig. 4.37. A). The N-terminus was not modelled, due to the absence of a structural template. Our proposal of IFS´s association to the ER membrane is also supported by the electrostatic distribution on the protein computed by the linear Poisson-Boltzmann equation (Fig. 4.37. B).

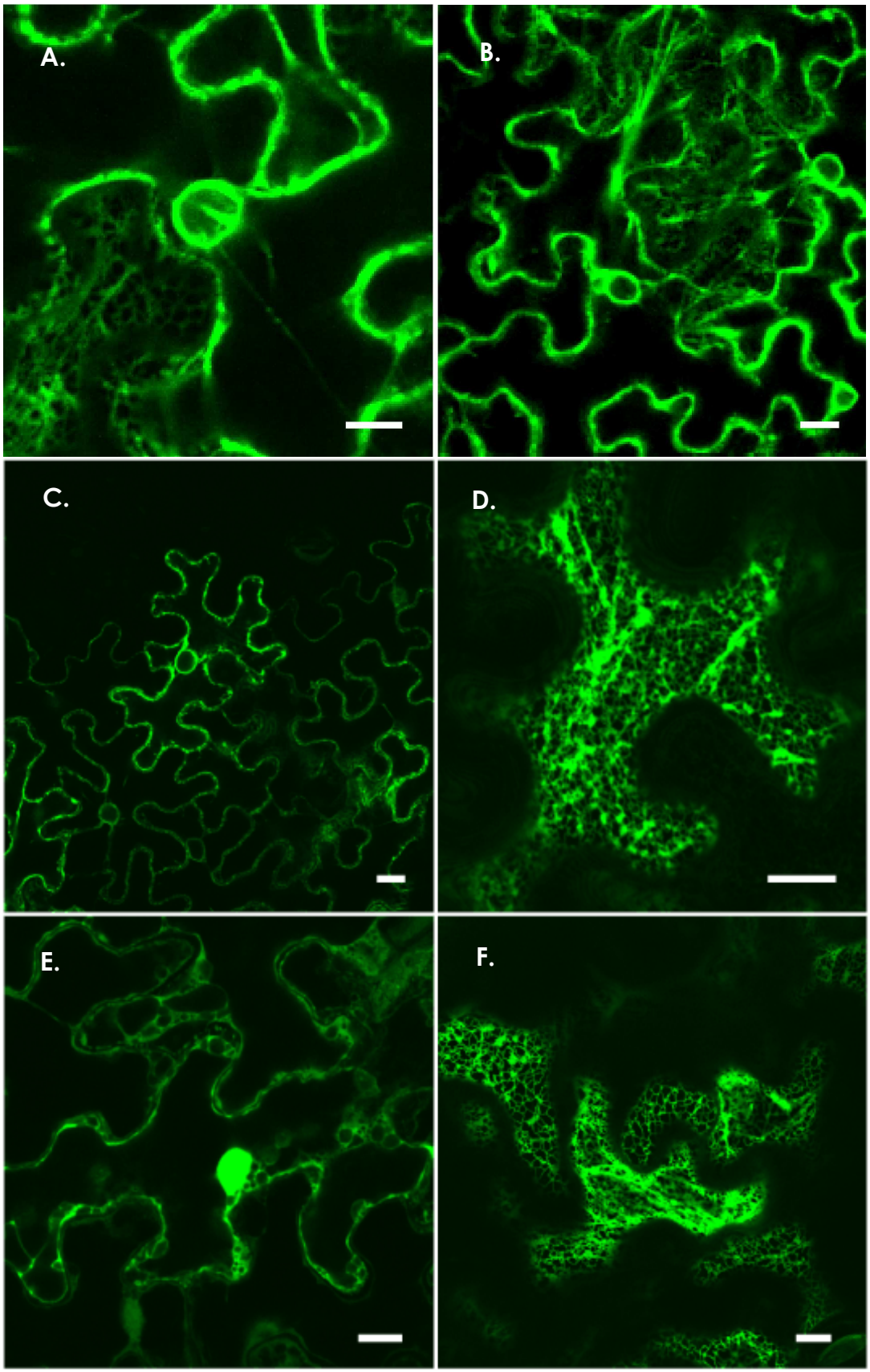


Fig. 4.36. Transient expression of 35::CYP93C18::GFP fusion proteins (A-D), of the control GFP itself (E) and of the GFP with HDEL sequence (F), in the lower epidermal cells of *Nicotiana benthamiana* leaves. The IFS::GFP fusion signal was observed within the endoplasmic reticulum, as predicted. Images were obtained by confocal microscopy [scale: bar =10 µm].

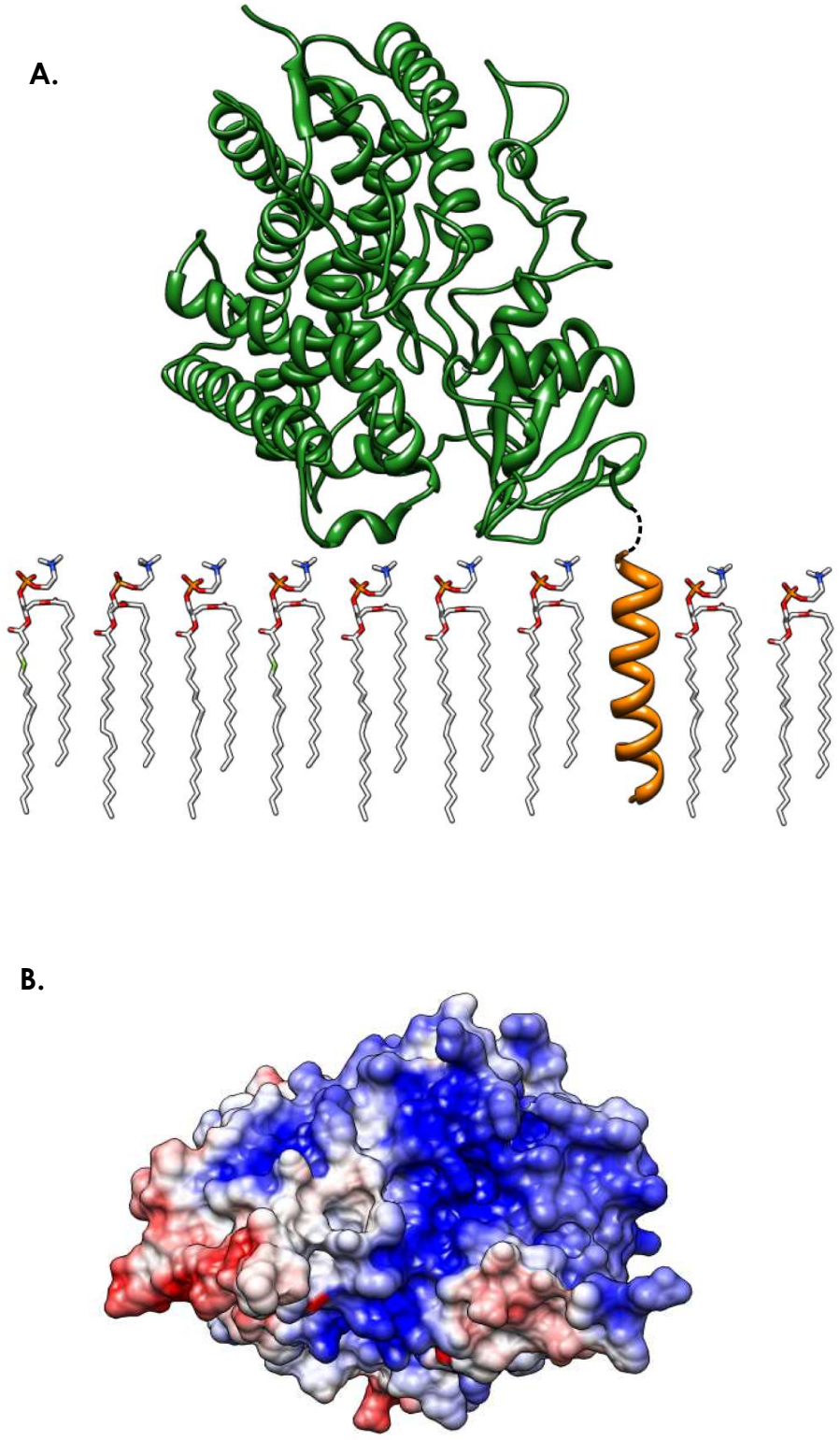


Fig. 4.37. (A) *In silico* modelled 3D structure of CYP93C18 and its association with the endoplasmic reticulum membrane on the cytosolic side. The N-terminal hydrophobic helix (indicated in orange) is anchored in the membrane. (B) Electrostatic distribution on the CYP93C18 model, viewed from the bottom side. Red colour indicates -5 kT/e, blue colour stands for +5 kT/e. The positively-charged and almost flat region represents a putative membrane association site.

5. DISCUSSION

The two main objectives of the present masters thesis were: (1) To identify isoflavone synthase genes in the selected non-leguminous and leguminous plant species, and (2) to perform a pilot study with IFS (CYP93C18) from *Pisum sativum* L. in order to test the feasibility of the functional expression for newly-identified isoflavone synthase genes and, moreover, to localize the protein *in vivo*. Our methodology was inspired by several successful studies reported in the literature (Jung *et al*., 2000; Akashi *et al*., 1999; Kim *et al*., 2003), which described the identification, cloning and functional expression of IFS from different leguminous species, in connection with the potential metabolic engineering of isoflavonoid biosynthesis.

5.1. *IFS* was detected in leguminous species only

Given that there are 60 isoflavonoid-producing plant families known to date (Lapčík, 2007), our approach was informed by five particular hypotheses: (1) The number of families producing isoflavonoids might in fact be significantly larger than hitherto thought, (2) plants producing isoflavonoids must necessarily possess an enzyme catalyzing the migration of the aryl group on the chromene skeleton of flavanones, (3) genes encoding for this enzyme should display a homology with genes for IFS already described in several leguminous species, (4) the absence of such orthologues need not always signify the absence of the biosynthetic pathway leading to the production of isoflavonoids – and thus the possible existence of some other, hitherto undiscovered enzyme(s) should not be ruled out, and, (5) conversely, the presence of an orthologue to CYP93C need not necessarily guarantee the presence of isoflavonoids, despite its high probability.

It must be admitted that our efforts towards the aim of identifying *IFS* in non-leguminous species did not yield fruit. Isoflavone synthase was discovered in none of the 33 examined non-leguminous species from 14 different plant families, although, as reported in the literature, in most of them isoflavonoids have been detected. Using the PCR method with degenerate primers, PCR products were obtained in 24 of the selected plants, once reaction conditions had been optimised. Even though the *IFS* fragment sequence determined by the degenerate primers was known from multiple alignment

analysis, there was no particular expectation as to the precise length of the PCR products, as the genomes of the examined plants are unknown (the variable constituent was primarily the intron length). The length of the amplified fragments obtained ranged from 550 bp to 1500 bp. In total, 28 sequences were acquired upon sequencing (some of which were fragments of different lengths originating from the same species). A BLAST search showed that half of them were not homologous to any nucleotide sequence from the database, and that the remaining sequences displayed homology with transposons and retrotrasposons, with fragments from mitochondrial and chloroplast genomes, and with various chromosomal DNAs not specified in any further detail. Moreover, when *CYP93C18*-specific primers were applied in the case of *Humulus lupulus* and *Iris* sp., PCR products of the promising lengths of 1700 bp (equal to the length of *IFS* from the *Pisum*) and 1500 bp, respectively, appeared. Surprisingly, the sequencing results were quite disappointing: the sequences did not share homology to any known *IFS* sequence from the GenBank.

These results contrast with the fact that IFS has been described in the case of one particular non-leguminous species once in the literature: Jung *et al*. identified two isoflavone synthase isoforms in *Beta vulgaris* L. from the family Chenopodiaceae (Jung *et al*., 2000), which is consistent with the presence of isoflavonoids in this plant (Geigert *et al*., 1973). However, the unexpectedly high homology of even the nucleotide sequences of these two genes with their orthologues from evolutionary distant leguminous plants (99% in most cases) does raise some awkward questions, even though the necessity for the protein sequence to have conserved regions is not open to doubt. *Beta vulgaris* was also examined in our study, but with a negative result, arising from repeated erroneous sequencing – which makes this result impossible to interpret.

On the other hand, this study demonstrates the utility of an undemanding PCR method with appropriately-designed primers to identify new isoflavone synthase genes in genomic DNAs of leguminous species. In control leguminous plants – *Phaseolus vulgaris* L. and *Pachyrhizus tuberosus* (Lam.) Spreng – we obtained new partial *IFS* sequences using the same, above-mentioned degenerate primers, and subsequently obtained also their complete *IFS* sequences, using consensual primers spanning the whole reading frame. The complete *IFS* sequences were 1860-bp long (including intron) in the case of *Phaseolus*, and 1803-bp long (including intron) in the case of *Pachyrhizus*. The introns revealed on the basis of alignment with the most homologous *IFS*s from GenBank, were 264-bp and 243-bp long, respectively. This means that they

were slightly longer than introns in soybean *IFS*1 (218 bp) and *IFS*2 (135 bp; Jung *et al*., 2000), and considerably longer than the 87-bp intron detected in *CYP93C18* in our study (see section 4.2.1.). The new *IFS* gene from *Phaseolus* displayed a very high degree of homology with *IFS*1 from *Vigna unguiculata* (L.) Walp at both DNA and protein levels (more than 97% of the amino-acids were similar). These findings correspond to the phylogenetical proximity of these two species within the millettioid clade of the Phaseoleae tribe (Cannon *et al.,* 2009). In the case of *Pachyrhizus*, the nucleotide sequence was most similar to the *IFS* from *Pueraria montana* var. *lobata*, both belonging to the Glycininae subtribe (Lee and Hymowitz, 2001). Surprisingly, its amino-acid sequence was highly homologous to IFS2 from *Vigna unguiculata* (L.) Walp., whose IFS was longer by three amino-acids. These differences between the DNA´s and the derived protein´s homologies could be explained by the manner in which the BLAST algorithm compares the sequence of interest with the database, without taking into account the degeneracy of the genetic code. The slightly higher percentage of identities in the case of the *IFS* gene from *Pueraria* versus the *IFS* gene from *Vigna* (89% versus 86% of identities), was not reflected in a higher percentage of identities in the derived amino-acid sequence in *Puearia*. In contrast, the amino-acid sequence of IFS2 from *Vigna*, belonging also to the Phaseoleae tribe but not in the Glycininae subtribe (Lee and Hymowitz, 2001), was found to be the most similar to our sequence. This could be due to the fact that not all mismatches in the nucleotide sequence necessarily affect the protein sequence.

The next appropriate step would be the verification of the the correct function of the newly-identified *IFS*s from *Phaseolus vulgaris* and *Pachyrhizus tuberozus*, using the methodology which was developed and successfully applied in the pilot study with CYP93C18, as descibed below.

However, our best efforts notwithstanding, the objective of identifying the *IFS* gene in non-leguminous plants was not met, as the methodology did not work here. This fact needs to be interpreted with caution. In particular: (1) In some of the plants investigated, isoflavone synthesis is indeed absent, and thus the gene itself is likely to be absent. (2) Primers designed on the basis of our knowledge of the *IFS* genes from leguminous plants and from sugar beet (*Beta vulgaris*), might not necessarily be appropriate to phylogenetically distant plant species. (3) It is improbable, yet possible, that one or more hitherto unknown proteins – responsible for isoflavone synthesis

through a different enzyme pathway – may exist instead of IFS, and if so, this pathway still awaits discovery.

5.2. CYP93C18 is a functional isoflavone synthase

The primary aim of the second part of this study was to develop an assay for the functional expression of IFS, using CYP93C18 as an example. But the study could be also considered as a continuation of the only study involving CYP93C18: in 2005, Cooper *et al.* demonstrated the obvious connection between up-regulation of the newly-discovered CYP93C18, "a putative isoflavone synthase gene", and a subsequent increase in isoflavonoid pisatin content in pea pods treated with insect elicitor (Cooper *et al*., 2005). However, the direct verification of the correct function of *CYP93C18* has never been reported.

In the present work, a successful pilot study with IFS from *Pisum sativum* L. (CYP93C18) has been carried out. The CYP93C18 was identified, cloned using Gateway™ Technology and introduced into the model plant *Arabidopsis thaliana* (L.) Heynh. ecotype Col-0.

Concerning our and Cooper´s CYP93C18 protein sequence, the nine differences discovered – all of which were upstream of the intron – could be ascribed to a genomic polymorphism of individual varieties of *Pisum sativum* L., as well as to possible sequencing errors in our and/or Cooper´s sequence. Moreover, a multiple alignment of our *CYP93C18* with the 30 known isoflavone synthase genes, including that described by Cooper (P450 Engineering database, University of Stuttgart), revealed that five of the mismatched amino-acids in our sequence corresponded to the consensus; three of the mismatches in Cooper´s sequence corresponded to the consensus; and both in Cooper's case and in ours, there was a mismatch at position 155 that did not correspond to the consesus. These findings support the polymorphism theory, and thus the mismatches are unlikely to be attributable to sequencing errors. Such errors can be ruled out as, in our case, the gene was sequenced three times from both 5´and 3´ends (each sequence run was based on an independent amplification reaction). In any case, the ascertained mismatches were not localized in the catalytic centre of the enzyme and did not include the conserved amino-acid residues Ser 310, Lys 375 and Leu 371, critical for aryl migration (Sawada *et al*., 2002; Sawada and Ayabe, 2005). Thus they had no influence over the localization and function of IFS, as was subsequently demonstrated in the present study.

In our work it was experimentally proved that the *CYP93C18* gene is present in the transgenic *Arabidopsis* genome, and that this gene was transcribed under the constitutive promoter CaMV35S, as confirmed by means of RT-PCR.

However, the proof of the correct gene expression, i.e. detection of CYP93C18 protein using western blot analysis, was sligthly equivocal because of the fact that no discrete band of the IFS appeared – due to the non-specificity of the antibody used. The CYP93C18-specific antibody conjugated with KLH, was designed according to Yu *et al*. (2000), who immunochemically detected the soybean IFS in microsomes prepared from transgenic tobacco leaves. The identical antibody was also employed for the same purpose in the study by Indu Jaganath (Jaganath, 2005), in which she attempted to detect the soybean IFS in both total and membrane proteins, from transgenic *Arabidopsis* (Col-0) leaves. As in the case of our work, she observed strong cross-reactivity of the antibody with proteins of various molecular sizes, although the IFS of 59 kDa in the protein extracts from soybean (a positive control) was clearly detected. In our case, the antibody bound to various proteins both from transgenic *Arabidopsis* and control plants (*Pisum* and wild-type *Arabidopsis*).

There are several explanations for this phenomenon: (1) The total protein extract, but not the membrane protein extract, was examined. The amount of protein which could cross-react was thus increased. Moreover, the prominent component of the leaf total protein extract is Rubisco large subunit of 55 kDa, whose preponderance in a sample could "overlay" some non-abundant proteins with similar molecular weight, possibly including also IFS. (2) The primary polyclonal antibody was produced in an immunized rabbit, and rabbits, being herbivorous animals, can often produce antisera displaying a non-specific background reaction against plant antigens (Oulehlová *et al*., 2009). (3) Peptides similar to that chosen by us for antibody design, can be found in a small number of other plant cytochromes P450, even though none of these peptides are identical to ours. (4) Most probably, polyclonal antibodies against the KLH:peptide conjugate can cross-react with various plant antigens in western blot analysis, thus causing the non-specificity of the immunodetection (Oulehlová *et al*., 2009). This system seems to be unsuitable when a particular protein from *Arabidopsis* or from *Pisum* is to be detected. Apart from this, the visualization system of the secondary Anti-rabbit IgG conjugated with alkaline phosphatase, was found to be less than ideal, as it is almost impossible to gain a sufficiently distinct digital image of the weakly-visualized proteins.

HPLC-MS analysis of the metabolites, however, was more relevant, as it provided the key proof of the correct function of CYP93C18 in the transgenic *Arabidopsis*. Compared to the 15 isoflavonoid standards, isoflavonoids genistein and tectorigenin were detected in transgenic *Arabidopsis* shoots and seeds, respectively, whereas no isoflavonoids were found in wild-type *Arabidopsis*. The latter finding reflects the fact that the gene orthologous to isoflavone synthase is not present within the *Arabidopsis* genome. Interestingly, the transformed *Arabidopsis* was able to produce small amounts of isoflavonoids without any previous stress induction such as UV-B treatment (Yu *et al*., 2000) or elicitation with yeast extract (Akashi *et al*., 1999), which are known to activate the phenylpropanoid pathway and increase the levels of isoflavonoids and of their precursor naringenin.

The present study thus provides the ample evidence that:

(1) CYP93C18 is a functional isoflavone synthase. Although only small traces of genistein and tectorigenin could be detected in IFS-transformed *Arabidopsis*, their presence was indisputable.

(2) *Arabidopsis* is an appropriate plant-expression system which disposes the IFS substrate naringenin. The elimination of water to yield isoflavones from 2-hydroxyisoflavanone is likely to be spontaneous; or the dehydratation could possibly be catalysed by 2-hydroxyisoflavanone dehydratase (Akashi *et al*., 2005), although no evidence of the presence of this enzyme in non-leguminous species has been reported, as far as we are aware. Moreover, isoflavone-modifying enzymes, namely hydroxylase and *O*-methyltransferase, clearly had to be present and be able to recognise the novel substrate genistein, as indicated by the presence of tectorigenin (4´,5,7-trihydroxy-6-methoxyisoflavone). Our results also indicate that the introduced IFS successfully competed with the endogenous enzymes involved in flavonoid biosynthesis, which also utilize the flavanone substrate naringenin – for instance, flavanone-3-hydroxylase or flavone synthase (Yu and McGonigle, 2005). Moreover, the surprising presence of the methylated isoflavonoid tectorigenin, a well-known anti-cancerogenic isoflavonoid (Thelen *et al*., 2005), in IFS-transgenic *Arabidopsis* is to the best of our knowledge reported here for the first time.

(3) Our simple methodology was proven to be very feasible for the exploration of the function of newly-identified genes for IFS from various species.

5.3. IFS is localized on the membrane of the endoplasmic reticulum

In addition, the predicted N-terminal signal peptide of CYP93C18, and thereby the latter´s ER localization, were verified by means of transient expression of CYP93C18::GFP in the tobacco leaves, where the GFP signal appeared on the endoplasmic reticulum, as predicted. This result is consistent with the concept of the isoflavonoid metabolone (Yu and McGonigle, 2005), where membrane-bound IFS ensures the correct metabolic channelling by interacting with enzymes upstream and downstream in the pathway. It is encouraging to note that a result similar to ours appears to have been obtained by Liu and Dixon (2001), albeit with IFS cDNA from *Medicago truncatula* overexpressed in *Medicago sativa* seedlings.

Our results, however, contrast with the conclusions reached by Indu Jaganath (cited by Crozier *et al*., 2006), who failed in her attempt to engineer the genistein metabolic pathway into *Arabidopsis* Col-0 using soybean IFS. One of the possible explanations the authors suggested for this failure entails considerations of IFS subcellular localization. Accordingly, the introduced *IFS* was transcribed at high levels but genistein was not produced, due to the potential mislocalization of IFS in the cytoplasm instead of its association with the ER membrane. This explanation was consistent with the fact that IFS was not detected by western blot analysis of membrane-bound proteins from transgenic lines. The question then arises as to why the IFS examined by them might have been incorrectly localized in the transgenic plants, whereas our IFS, as well as those by other investigators (Jung *et al.*, 2000; Yu *et al*., 2000 and others), were indeed correctly embedded in the membrane.

The manner in which the IFS is attached to the ER membrane is still not entirely clear. It is generally acknowledged that membrane-bound P450s have their N-terminal signal sequence anchored in the membrane and hydrophobic residues in the F-G loop (between F and G helices) associated with the cytosolic side of the ER membrane (Baudry *et al.*, 2006). Other possible interaction domains, such as segments before and after the A-helix, and amino-acid residues in β strand 2-2, were also described (Williams *et al*., 2000).

Our *in silico* predictions involving isoflavone synthase, and the model generated are consistent with the above-mentioned features of the membrane-bound P450s. In a manner analogous to Dai *et al.*, who constructed a model of mammalian P450 2B1 and clarified its mode of attachement to the endoplasmic reticulum membrane (Dai *et al*.,

1998), we propose a similar mode of IFS membrane insertion. The N-terminus was predicted as the only transmembrane domain of the protein. Additional hydrophobic regions, localized on the same protein face as the N terminus and protruding from the protein surface, could be found within the F-G loop, the pre-A region as well as in the β sheet 2-2. This proposed IFS membrane association, however, remains to be confirmed, by means of the methods of structural biology.

Taken together, our data provide direct evidence for the proposed isoflavone synthase enzymatic activity of the *Pisum sativum* L. CYP93C18 protein *in vivo*. Moreover, we demonstrated that the isoflavone synthase from pea is associated with the endoplasmic reticulum membrane on its cytosolic side and, according to our hypothetical model, it is likely to be anchored there by the N-terminal transmembrane domain.

6. CONCLUSIONS

The present masters thesis dealt with the genetic background of the biosynthesis of plant secondary metabolites – isoflavonoids, specifically with the genes for the isoflavone synthase (IFS) – a key enzyme in the isoflavonoid biosynthetic pathway.

In the light of the current state of knowledge, it was attempted to identify isoflavone synthase genes in 33 species from 14 different families of non-leguminous plants, using the PCR method with degenerate primers. In spite of the considerable effort expended, no such gene was identified in this study. However, the method worked in the two control leguminous species, *Phaseolus vulgaris* L. and *Pachyrhizus tuberozus* (Lam.) Spreng., whose new complete genes for *IFS*, homologous to known *IFS* genes in GenBank, were thus obtained. As a project for the future, it will be appropriate to confirm the correct function of these new genes by means of functional expression.

A pilot study with IFS from *Pisum sativum* L. (CYP93C18) was successfully conducted. The *CYP93C18* gene was identified, cloned and introduced into the isoflavone pathway-free plant *Arabidopsis thaliana*, using Gateway^{TM} Technology. The correct function of the gene was verified at four different levels and the most important result to emerge was the detection of the isoflavonoids genistein and tectorigenin in the transgenic *Arabidopsis*. The methodology used is thus applicable to the study of the function of newly-identified genes for IFS from other species also.

In addition, CYP93C18::GFP fused proteins were transiently expressed in the leaves of *Nicotiana benthamiana*, and the localization of the GFP signal was observed on the endoplasmic reticulum, using confocal microscopy. This was consistent with data previously reported in the literature and with the *in silico* predictions. The mode of the attachement of IFS to the endoplasmic reticulum membrane suggested in our model, however, still remains to be experimentally clarified using methods of structural biology.

7. REFERENCES

Akashi T., Aoki T., Ayabe S. (1999): Cloning and functional expression of a cytochrome P450 cDNA encoding 2-hydroxyisoflavanone synthase involved in biosynthesis of the isoflavonoid skeleton in licorice. Plant Physiol. 121: 821–828.

Akashi T., Aoki T., Ayabe S. (2005): Molecular and biochemical characterization of 2-hydroxyisoflavanone dehydratase. Involvement of carboxylesterase-like proteins in leguminous isoflavone biosynthesis. Plant Physiol. 137: 882–891.

Akashi T., Sawada Y., Aoki T., Ayabe S. (2000): New scheme of the biosynthesis of formononetin involving 2,7,4'-trihydroxyisoflavanone but not daidzein as the methyl acceptor. Biosci. Biotechnol. Biochem. 46: 2276-2279.

Aksamit-Stachurska A., Korobczak-Sosna A., Kulma A., Szopa J. (2008): Glycosyltransferase efficiently controls phenylpropanoid pathway. BMC Biotechnol. 8: 25.

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J, Zhang Z., Miller W., Lipman D. (1997): Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Ayabe S. and Akashi T. (2006): Cytochrome P450s in flavonoid metabolism. Phytochem. Rev. 5: 271-282.

Baker N.A., Sept D., Joseph S., Holst M.J., McCammon J.A. (2001): Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl. Acad. Sci. USA 98: 10037-10041.

Baudry J., Rupasinghe S., Schuler M.A. (2006): Class-dependent sequence alignment strategy improves the structural and functional modeling of P450s. Protein Eng. Des. Sel. 19(8): 345–353.

Bendtsen J.D., Nielsen H., Von Heijne G., Brunak S. (2004): Improved prediction of signal peptides: SignalP3.0. J. Mol. Biol. 340: 783-795.

Bennetts H.W., Uuderwood E.J, Shier F. L. (1946): A specific breeding problem of sheep on subterranean clover pastures in western australia**.** Aust. Vet. J. 22 (1): 2–12.

Boker L.H., Van der Schouw Y.T., De Kleijn M.J.J., Jacques P.F, Grobbee D.E., Peeters P.H.M. (2002): Intake of dietary phytoestrogens by Dutch women. J. Nutr. 132: 1319-1328.

Boland M. and Donnelly D.M.X. (1998): Isoflavonoids and related compounds. Nat. Prod. Rep. 15(3): 241-260.

Bolwell G.P., Bozak K., Zimmerlin A. (1994): Plant cytochrome P450. Phytochem. 37(6): 1491-506.

Broughton W.J., Jabbouri S., Perret X. (2000): Keys to symbiotic harmony. J. Bact. 182(20): 5641-5652.

Bryson K., McGuffin L.J., Marsden R.L., Ward J.J., Sodhi J.S., Jones D.T. (2005): Protein structure prediction servers at University College London. Nucleic Acids Res. 33: W36-38.

Brzezinski A. and Debi A. (1999): Phytoestrogens: the 'natural' selective estrogen receptor modulators? Eur. J. Obstet. Gynecol. Reprod. Biol. 85: 47–51.

Buchanan B.B., Gruissem W., Jones. R.L. (2002): Biochemistry & molecular biology of plants. American Society of Plant Physiologists, 1304-1306.

Cannon S.B., May G.D., Jackson S.A. (2009): Three sequenced legume genomes and many crop species: rich opportunities for translational genomics. Plant Physiol. 151: 970-977.

Clough S.J., Bent A.F. (1998): Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16(6): 735-743.

Cooper E.J. (2004): Multiple responses of rhizobia to flavonoids during legume root infection. Adv. Bot. Res. 41**:** 1–62.

Cooper L.D., Doss R.P., Price R., Peterson K., Olivern J.E. (2005): Application of Bruchin B to pea pods results in the up-regulation of CYP93C18, a putative isoflavone synthase gene, and an increase in the level of pisatin, an isoflavone phytoalexin. J. Exp. Bot. 56(414): 1229–1237.

Cornwell T., Cohick W, Raskin I. (2004): Dietary phytoestrogens and health. Phytochem. 65: 995–1016.

Crozier A., Jaganath I.B., Clifford M.N. (2006): Phenols, polyphenols and tannins: An overview. In: Plant secondary metabolites: Occurrence, structure and role in the human diet. Blackwell Publishing Ltd., 1-24.

Dai R, Pincus MR, Friedman FK (1998): Molecular modeling of cytochromes P450 2B1: Mode of membrane insertion and substrate specifity. J. Protein Chem. 17(2):121-129.

Dakora F.D. and Phillips D.A. (1996): Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. Physiol. Mol. Plant Pathol. 49: 1-20**.**

Deavours B.E. and Dixon R.A. (2005): Metabolic engineering of isoflavonoid biosynthesis in alfalfa. Plant Physiol 138: 2245-2259.

Demmig-Adams B. and McCauley L. (2005): Breast cancer, estrogen, soy genistein, and other dietary factorsTowards an understanding of their mechanistic interactions. Nutrition & Food Science 35(1): 35-42.

Dhaubhadel S., Farhangkhoee M. and Chapman R. (2008): Identification and characterization of isoflavonoid specific glycosyltransferase and malonyltransferase from soybean seeds. J. Exp. Bot. 59(4): 981-994.

Dixon R.A. and Steele C.L. (1999): Flavonoids and isoflavonoids-a gold mine for metabolic engineering. Trends Plant Sci. 4(10): 394-400.

Du H., Huang Y., Tang Y. (2010): Genetic and metabolic engineering of isoflavonoid biosynthesis. Appl. Microbiol. Biotechnol. 86: 1293–1312.

Duffy C., Perez K., Partridge A. (2007): Implications of phytoestrogen intake for breast cancer. CA Cancer J. Clin. 57: 260-277.

Eswar N., Marti-Renom M. A., Webb B., Madhusudhan M. S., Eramian D., Shen M., Pieper U., Sali A. (2006): Comparative protein structure modeling with Modeller. Cur. Prot. Bioinf. Supplement 15: 5.6.1-5.6.30.

Fisher R.F. and Long S.R. (1993): Interactions of NodD at the nod Box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. J. Mol. Biol. 233(3): 336-48.

Gavaler J.S., Rosenblum E.R., Van Thiel D.H., Eagon P.K., Pohl C.R., Campbell I.M., Gavaler J. (1987): Biologically active phytoestrogens are present in bourbon. Alcohol Clin. Exp. Res. 11: 399-406.

Geigert J., Stermitz F. R., Johnson G., Maag D. D., Johnson D. K. (1973): Two phytoalexins from sugarbeet (*Beta vulgaris*) leaves. Tetrahedron 29(18): 2703-2706.

Gnanamanickam S.S. (1979): Isolation of isoflavonoid phytoalexins from seeds of Phaseolus vulgaris L. Experientia 35: 323.

Hagmann M. and Grisebach H. (1984): Enzymatic rearrangement of flavanone to isoflavone. FEBS Lett. 175(2): 199-202.

Hakamatsuka T., Hashim M.S., Ebizuka Y., Sankawa U. (1991): P-450-dependent oxidative rearrangement in isoflavone biosynthesis: reconstitution of P-450 and NADPH:P450 reductase. Tetrahedron 47: 5969-5978.

Hakamatsuka T., Mori K., Ishida S., Ebizuka Y. , Sankawa U. (1998): Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of Pueraria lobata in honour of Professor G. H. Neil Tower´s 75th Birthday. Phytochem. 49(2): 497-505.

Haseloff J., Siemering K.R., Prasher D.C., Hodge S. (1997): Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. USA 94:2122–2127.

Hashim M.F., Hakamatsuka T., Ebizuka Y., Sankawa U. (1990): Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis. FEBS Lett. 271 (1-2): 219-222.

Hofmann K., Stoffel W. (1993): TMbase - A database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seyler 374: 166.

Jacobs M. and Rubery P.H. (1988): Naturally occurring auxin transport regulators. Science 341(4863): 346-349.

Jaganath I.B. (2005): Dietary flavonoids: Bioavailabilty and biosynthesis. PhD thesis, University of Glasgow.

Jung W., Yu O., Lau S.C., O´Keefe D., Odell J., Fader G. McGonigle B. (2000): Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat. Biotechnol. 18: 208–212.

Kim B.G., Kim S-Y., Song H.S., Chan Lee CH., Hur H-G., Kim S-I., Ahn J.H. (2003): Cloning and expression of the isoflavone synthase gene (IFS-Tp) from Trifolium pratense. Mol. Cells 15(3): 301-306.

Kochs G. and Grisebach H. (1986): Enzymic synthesis of isoflavones. Eur. J. Biochem. 155(2): 311-318.

Kurzer M.S. and Xu X. (1997): Dietary phytoestrogens. Annu. Rev. Nutr. 17: 353-81.

Lackhan N.K. (1994): Investigations on in vitro production of rotenone using Pachyrhizus erosus (L.) Urban. MPh thesis, University of St. Augustine.

Lapčík O. (2004): Endocrinological aspects of dietary habits. Czech J. Food Sci. 22: 29-38.

Lapčík O. (2007): Isoflavonoids in non-leguminous taxa: A rarity or rule? Phytochem. 68(22-24): 2909-2916.

Lapčík O., Hill M., Hampl R., Wähälä K., Adlercreutz H. (1998): Identification of isoflavonoids in beer. Steroids 63: 14–20.

Lapčík O., Hill M., Černý I., Lachman J., Al-Maharik N., Adlercreutz H., Hampl R. (1999): Immunoanalysis of isoflavonoids in *Pisum sativum* and *Vigna radiata*. Plant Sci. 148: 111–119.

Lapčík O., Vítková M., Klejdus B., Al-Maharik N., Adlercreutz H. (2004): Immunoassay for biochanin A. J. Immunol. 294(1-2): 155-63.

Li J.F., Park E., Von Arnim A.G., Nebenführ A. (2009): The FAST technique: a simplified Agrobacterium-based transformation method for transient gene expression analysis in seedlings of Arabidopsis and other plant species. Plant Methods 5: 6.

Liu C.J., Blount J.W., Steele C.L., Dixon R.A. (2002): Bottlenecks for metabolic engineering of isoflavone glycoconjugates in Arabidopsis. Proc. Natl. Acad. Sci. USA. 99(22): 14578-14583.

Liu CH-J. and Dixon R.A. (2001): Elicitor-induced association of isoflavone *O*-methyltransferase with endomembranes prevents the formation and 7-*O*-methylation of daidzein during isoflavonoid phytoalexin biosynthesis. Plant Cell 13: 2643–2658.

Liu R., Hu Y., Li J., Lin Z. (2007): Production of soybean isoflavone genistein in non-legume plants via genetically modified secondary metabolism pathway. Metab. Eng. 9(1): 1-7.

Macková Z., Koblovská R., Lapčík O. (2006): Distribution of isoflavonoids in non-leguminous taxa – An update. Phytochem. 67: 849–855.

Mansuy D. (1998): The great diversity of reactions catalyzed by cytochromes P450. Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 121(1-3): 5-14.

Mazur W. and Adlercreutz H. (1998): Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment. Naturally occurring oestrogens in food. Pure Appl. Chem. 70: 1759–1776.

Mazur W. M., Wihala K., Rasku S., Salakka A., Hase T., Adlercreutz H. (1998): Lignan and isoflavonoid concentrations in tea and coffee. Br. J. Nutr. 79: 37-45.

Misra P., Pandey A., Tewari S.K., Nath P., Trivedi P.K. (2010): Characterization of isoflavone synthase gene from Psoralea corylifolia: a medicinal plant. Plant Cell Rep. 29(7):747-755.

Mousavi Y. and Adlercreutz H. (1993): Genistein is an effective stimulator of sex hormone-binding globulin production in hepatocarcinoma human liver cancer cells and suppresses proliferation of these cells in culture. Steroids 58(7): 301-304.

Nakagawa T., Kurose T., Hino T., Tanaka K., Kawamukai M., Niwa Y., Toyooka K., Matsuoka K., Jinbo T., Kimura T. (2007): Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J. Biosci. Bioeng. 104(1): 34-41.

Nakamura Y., Tsuji S., Tonogai Y. (2000): Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily intake. J. AOAC Int. 83(3): 635-650.

Ososki A.L. a Kennelly E.J. (2003): Phytoestrogens: a Review of Present State of research. Phytoter. Res. 17: 845-869.

Oulehlová D., Hála M., Potocký M., Žárský V., Cvrčková F. (2009): Plant antigens cross-react with rat polyclonal antibodies against KLH-conjugated peptides. Cell Biol. Internat. 33:113-118

Overkamp S., Hein F., Barz W. (2000): Cloning and characterization of eight cytochrome P450 cDNAs from chickpea (Cicer arietinum L.) cell suspension cultures. Plant Sci. 12(155): 101-108.

Pettersen E.F., Goddard T.D., Huang C.C., Couch G.S., Greenblatt D.M., Meng E.C., Ferrin T.E. (2004): UCSF Chimera – a visualization system for exploratory research and analysis. J. Comput. Chem. 25(13): 1605-1612.

Ravichandran K.G., Boddupalli S.S., Hasemann C.A., Peterson J.A., Deisenhofer J. (1993): Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450s. Science 261: 731-736.

Reynaud J., Guilet D., Terreux R., Lussignol M., Walchshofer N. (2005): Isoflavonoids in non-leguminous families: an update. Nat. Prod. Rep. 22**:** 504–515.

Rosenblum E.R., Campbell I.M., Van Thiel D.H., Gavaler J.S. (1992): Isolation and identification of phytoestrogens from beer. Alcohol Clin. Exp. Res. 16: 843-845.

Sawada Y. and Ayabe S. (2005): Multiple mutagenesis of P450 isoflavonoid synthase reveals a key active-site residue. Biochem. Bioph. Res. Co. 330: 907–913.

Sawada Y., Kinoshita K., Akashi T., Aoki T., Ayabe S. (2002): Key amino acid residues required for aryl migration catalysed by the cytochrome P450 2-hydroxyisoflavanone synthase. Plant J. 31: 555–564.

Schwab W. (2003): Metabolome diversity: too few genes, too many metabolites? Phytochem. 62: 837–849.

Shih Ch-H., Chen Y., Wang M., Chu I.K., Lo C. (2008): Accumulation of isoflavone genistin in transgenic tomato plants overexpressing a soybean isoflavone synthase gene. J. Agric. Food Chem. 56(14): 5655-61.

Shimada N., Aoki T., Sato S., Nakamura Y., Tabata S., Ayabe S. (2003): A cluster of genes encodes the two types of chalcone isomerase involved in the biosynthesis ofgeneral flavonoids and legume-specific 5-deoxy(iso)flavonoids in *Lotus japonicus*. Plant Physiol. 131: 941-951.

Siminszky B., Corbin F.T., Ward E.R., Fleischmann T.J., Dewey R.E. (1999): Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco

enhances the metabolism of phenylurea herbicides. Proc. Natl. Acad. Sci. USA. 96(4): 1750-1755.

Sreevidya V.S., Rao S.C., Sullia S.B., Ladha J.K., Reddy P.M. (2006): Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia. J. Exp. Bot. 57: 1957-1969.

Steele C.L., Gijzen M., Qutob D., Dixon R.A. (1999): Molecular characterization of the enzyme catalyzing the aryl migration reaction of isoflavonoid biosynthesis in soybean. Arch. Biochem. Biophys. 367: 146–150.

Subramanian S., Stacey G., Yu O. (2006): Endogenous isoflavonoids are essential for the establishment of symbiosis between soybean and Bradyrhizobium japonicum. Plant J. 48(2): 261-273.

Subramanian S., Stacey G., Yu O. (2007): Distinct, crucial roles of flavonoids during legume nodulation. Trends Plant Sci. 12(7) 282-285.

Subramanian S., Xu L., Lu G., Odell J., Yu O. (2004): The promoters of the isoflavone synthase genes respond differentially to nodulation and defense signals in transgenic soybean roots. Plant Mol. Biol. 54: 623–639.

Thelen P., Scharf J.G., Burfeind P., Hemmerlein B., Wuttke W., Spengler B., Christoffel V., Ringert R.H., Seidlová-Wuttke D. (2005): Tectorigenin and other phytochemicals extracted from leopard lily *Belamcanda chinensis* affect new and established targets for therapies in prostate cancer. Carcinogenesis 26(8): 1360-1367.

Tian L. and Dixon R.A. (2006): Engineering isoflavone metabolism with an artificial bifunctional enzyme. Planta 224(3): 496-507.

VanEtten H. D., Mansfield J. W., Bailey J. A., Farmer E.E. (1994): Two classes of plant antibiotics: Phytoalexins versus "Phytoanticipins". Plant Cell 6: 1191-1192.

Veitch N. (2007): Isoflavonoids of the Leguminosae. Nat. Prod. Rep. 24: 417–464.

Wang W., Vignani R., Scali M., Crest M. (2006): A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Electrophoresis 27(13): 2782 – 2786.

Weigel D. and Glazebrook J. (2002): Arabidopsis. A laboratory handbook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Williams P.A., Cosme J., Sridhar V., Johnson E.F., McRee D.E. (2000): Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity. Mol. Cell. 5:121-131.

Williams L.A. and Kane C.M. (1993): Direct sequencing of PCR products with degenerate primers. Promega Notes Magazine 40: 8-12.

Winkel-Shirley B. (2001): Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant physiol. 126: 485-493.

Yu O., Jung W., Shi J., Croes R.A., Fader G.M., McGonigle B., Odell J.T. (2000): Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. Plant Physiol. 124(2): 781-94.

Yu O. and McGonigle B. (2005): Metabolic engineering of isoflavone biosynthesis. Adv. Agr. 86: 147-190.